NOVEL ANTISENSE OLIGOMERS AND USE THEREOF

Field of the invention

The invention relates to methods for modulating gene expression by antisense technology. The invention relates in particular to the inhibition of matrix degradation in the treatment of osteoarthritis.

Background

Antisense technology has been used for a long time to inhibit the expression of genes. The design of antisense oligonucleotides has largely focused on pre-spliced mRNA and more in particular on the 5' and 3' splice junctions of an exon. The possibility that such oligonucleotides may also interfere with genomic DNA, and as a consequence reduce efficiency or have toxic effects to a cell, has not been considered in great detail up to now.

The success in inhibiting the splicing and translation of a gene by antisense technology is often variable and several antisense oligomers which span sections along the entire primary transcript have to be developed and tested in order to obtain an oligonucleotide which provides sufficient inhibition. Despite the widespread use of antisense technology there is still a need for more efficient antisense oligonucleotides.

Normal function of a synovial joint depends on proper functioning of all tissues in this organ. The loaded tissues in weight-bearing joints are articular cartilage and subchondral bone. Proper function of these tissues, e.g. deformation under load, shock absorption and lubrication of the load-bearing articular surfaces is dependent on the biochemical composition of their extracellular matrix (ECM). The homeostasis of this highly specialised ECM is controlled by the articular cartilage cell: the chondrocyte. In order to maintain an optimal ECM composition, chondrocytes constantly – and at random – replace the molecular constituents of their extracellular environment. To achieve this, auto/paracrine catabolic and anabolic growth factor pathways balance each other's activities. In other words, a constant turnover rate of the ECM is achieved when physiological degradative and

synthetic processes are in equilibrium. The actors of these auto/paracrine pathways, the growth factors, the cytokines and their respective plasma membrane receptors, are produced by the chondrocytes themselves.

In normal healthy cartilage, the Insulin-Like Growth Factor /Insulin-Like Growth Factor I Receptor (IGF1/IGF1R) pathway is responsible for the anabolic activities of the chondrocyte, while the Interleukin-1/Interleukin-1 Receptor type I (IL1/IL1RI) pathway controls chondrocyte catabolic action. Specific receptors that induce intracellular post-signalling activities are responsible for the activity of each pathway. IL1 activities are ensured by functional receptors and are controlled by the production of receptor antagonists or decoy receptors. IL1 (IL1-alpha (IL1A) and IL1-beta (IL1B)) transmits its signal through the cell after binding to the type 1 receptor for IL1 (IL1RI). The presence of a decoy type 2 receptor for IL1 (IL1RII) that is unable to transmit an intracellular signal after IL1 binds to it, offers a mechanism for the chondrocyte to remain in control of too abundant catabolic IL1 activity. IL1RII thus functions as a biological mousetrap for IL1. In normal healthy cartilage, IGF-1/IGF1R induced IL1RII overrules the catabolic IL1/IL1RI pathway and controls the homeostasis of the ECM (Wang et al. Arthritis Rheum (2003);48:1281-91)

Osteoarthritic (OA) cartilage is characterised by destructive processes, which is known to be a result of the disruption of the equilibrium between the IGF-1/IGF-1R and IL1/IL1RI pathways. Inhibition of the IL1/IL1RI pathway has been suggested using both IL1 inhibitors (such as antibodies) and IL1 receptor antagonists, with varying results.

Antisense inhibition of IL1 using antisense has been reported for hematopoietic cells *in vitro* (Manson et al. 1990, *Lymphokine Res.* 9: 35-42), but has not led to further investigations on *in vivo* therapeutic applications.

US patent 5,856,099 describes the use of oligonucleotides for the inhibition of IL1RI by antisense technology. The oligonucleotides described therein are complementary to regions in the unspliced mRNA. Oligonucleotides were designed

against the 5' Cap, 5'UTR, start AUG, splice junction regions containing both exon and intron sequence, exonic sequences, and 3' UTR region. Only a very limited number of the designed antisense nucleotides gave a significant inhibition of IL1RI expression (between 46 and 90 %). These oligonucleotides were directed against the 3' UTR of the prespliced mRNA of IL1RI. IC_{50} values for the optimal oligonucleotide were between 75 and 100 nM, depending on the cell type used. US 5,856,099 demonstrates that, using a classical design of antisense oligonucleotides for the antisense inhibition of IL1RI, a limited number of oligonucleotides are identified which are effective at concentrations of 100 nM of more.

Summary of the invention

The present invention presents a method of designing and using antisense oligomers that allow a more efficient modulation of a target gene. Oligomers are used which are complementary to the spliced mRNA and not to pre-spliced mRNA or genomic DNA. Specificity for spliced mRNA is achieved by targeting those regions in the spliced mRNA which encompass the junction of two consecutive exons obtained after the removal by splicing of at least one intron between two exons from the primary pre-spliced RNA transcript. The use of such antisense oligomers, herein referred to as exon-bridging oligomers, prevents aspecific binding of the oligomer to the genomic DNA of the targeted gene itself. Moreover, the present invention provides methods for further decreasing the interaction of the oligomers with any genomic DNA. Thus, the local distortion of the genome and activation or inhibition of other genes which are located on the genome in the vicinity of the target gene is avoided. Such unwanted gene activation/inhibition can cause undesirable side effects or cell toxicity. Because the exon-bridging oligomers are not scavenged by genomic DNA, the amount of exon-bridging antisense oligomer required to obtain effective inhibition is significantly reduced compared to conventional antisense oligomers. Thus the present invention relates to antisense oligonucleotides that can be used at lower concentrations, can

provide more efficient inhibition and are less likely to cause unwanted effects such as reduced viability of cells being treated with antisense oligonucleotides.

Thus, a first aspect of the present invention relates to exon-bridging probes, which are of use in different applications including, but not limited to the methods described herein, i.e. *in vitro* or *in vivo* modulation of the expression of a target gene in a cell population, more particularly inhibition of a target gene.

According a particular embodiment of the invention, the exon-bridging antisense oligomers of the invention have a length of between 15-30 nucleotides, particularly of 20 nucleotides. A further particular embodiment of the invention relates to exon-bridging antisense oligomers of which the complementary sequence has less than 70% sequence identity with a nucleotide sequence other than the mature mRNA or DNA of said target gene. In another particular embodiment, the invention provides exon-bridging antisense oligomers having a GC content of at least 45%. In another particular embodiment of the invention exon-bridging antisense oligomers are provided wherein the sequences complementary to the 5' and 3' end of the exon-exon boundary of the mRNA of said target gene within the exon-bridging antisense oligomer have a Tm of less than 32-36°C. Additionally or alternatively, the exon-bridging antisense oligomers of the invention do not comprise a sequence of more than 11 consecutive nucleotides which are complementary to the sequence at the 3' end or the sequence at the 5' end of the exon-exon boundary in the mature mRNA of the target gene. Moreover, additionally or alternatively according to the present invention, the exon-bridging antisense oligomers have a sequence which displays at least 70 % sequence identity with the complementary sequence of the cDNA of said target gene.

A further particular embodiment of this aspect of invention relates to exon-bridging antisense oligomers complementary to the mRNA of IL1RI for the inhibition of the expression of the IL1RI gene.

A particular embodiment of this aspect of the invention relates to exon-bridging antisense oligomers directed which bridge exon 02-03, which are particularly suited for use in the methods of the present invention. Thus in particular, the present invention relates to probes having at least 70% sequence identity with the complementary sequence of the cDNA sequence spanning exons 02-03. Even more particularly, the present invention provides an exon-bridging antisense oligomer selected from the group consisting of probe Nos. 6 (SEQ ID NO:6), 7 (SEQ ID NO:7), 8 (SEQ ID NO:8) and 21 (SEQ ID NO:21) described herein and exon-bridging antisense oligomers having at least 70% sequence identity with the sequence of probe NO. 6, 7, 8 or 21 described herein, more particularly exon bridging antisense oligomers which additionally have at least 70% sequence identity with the complementary sequence of the cDNA of the IL1R1 gene corresponding to probes NO.6, 7, 8, or 21.

Another particular embodiment of this aspect of the invention relates to exon-bridging antisense oligomers directed which bridge exon 05-06, which are particularly suited for use in the methods of the present invention. Thus in particular, the present invention relates to probes having at least 70% sequence identity with the complementary sequence of the cDNA sequence spanning exon 05-06. Even more particularly, the present invention provides an exon-bridging antisense oligomer selected from the group consisting of probe No 24 (SEQ ID NO:24) and exon-bridging antisense oligomers having at least 70% sequence identity with the sequence of probe NO. 24 described herein, more particularly exon bridging antisense oligomers which additionally have at least 70% sequence identity with the complementary sequence of the cDNA of the IL1R1 gene corresponding to probes NO. 24.

A second aspect of the present invention relates to a method for the *in vitro* or *in vivo* modulation of the expression of a target gene in a cell population with an antisense oligomer said method characterised in that mature mRNA function is inhibited by contacting the cells with an exon-bridging antisense oligomer directed against said mature mRNA.

According a particular embodiment of the invention, the exon-bridging antisense oligomers used in the method of the invention have a length of between 15-30 nucleotides, particularly of 20 nucleotides.

According a particular embodiment of the invention, the method comprises the step of contacting the cells with the exon-bridging oligomers in the absence of a DNA transfecting agent.

According a particular embodiment of the invention, a method for inhibiting a target gene is provided, wherein the function of all mature mRNAs originating from said target gene is inhibited using one or more exon-bridging antisense oligomers.

According to a particular embodiment of the invention, a method of inhibition is provided using exon-bridging antisense oligomers wherein the complementary sequence of the exon-bridging antisense oligomer has less than 70% sequence identity with a nucleotide sequence other than the mature mRNA or DNA of said target gene.

According to a particular embodiment of the invention, a method of inhibition is provided using an exon-bridging antisense oligomer wherein the exon-bridging antisense oligomer has a GC content of at least 45%.

According to a particular embodiment of the invention, a method of inhibition is provided using an exon-bridging antisense oligomer wherein the sequences complementary to the 5' and 3' end of the exon-exon boundary of the mRNA of said target gene within the exon-bridging antisense oligomer have a Tm of less than 36°C, particularly between 32-36°C.

According to a particular embodiment of the invention, a method of inhibition is provided using an exon-bridging antisense oligomer wherein the exon-bridging antisense oligomer does not comprise a sequence of more than 11 consecutive

nucleotides which are complementary to the sequence at the 3' end or the sequence at the 5' end of the exon-exon boundary in the mature mRNA of the target gene.

According to a particular embodiment of the invention, a method of inhibition is provided using an exon-bridging antisense oligomer wherein wherein the sequence of the exon-bridging antisense oligomer sequence has at least 70 % sequence identity with the complementary sequence of the cDNA of said target gene.

According to a particular embodiment of the invention, a method of inhibition is provided using an exon-bridging antisense oligomer, which comprises contacting the cells with 1 to 100 nM, particularly 1 to 10 nM, of the exon-bridging antisense oligomer.

According to a particular embodiment of this aspect of the invention, an *in vitro* or *in vivo* method for inhibition of IL1R1 expression is provided by using the exon-bridging antisense oligomers of the present invention directed against the IL1R1 mRNA.

According to a particular embodiment of this aspect of the invention, an *in vitro* or *in vivo* method for increasing the synthesis of extracellular matrix compounds in a cell population by inhibiting the expression of IL1RI is provided characterised in that it comprises the step of contacting the cells with an IL1RI exon-bridging antisense oligomer. More particularly, according to the invention, this can be achieved using the antisense oligomers which having a sequence of 15 to 30, more particularly 20 nucleotides, which are complementary to the sequence in the IL1R1 cDNA bridging exons 02-03 or 05-06. Most particularly, the present invention relates to a method of increasing the synthesis of ECM compounds by using probes selected from the group of probes Nos 6 (SEQ ID NO:6), 7 (SEQ ID NO:7), 8 (SEQ ID NO:8), 21 (SEQ ID NO:9) and 24 (SEQ ID NO:24) described herein or exon-bridging antisense oligomers having at least 70% sequence identity

with the sequence of probe Nos. 6, 7 8, 21 or 24 described herein, more particularly exon bridging antisense oligomers which additionally have at least 70% sequence identity with the complementary sequence of the cDNA of the IL1R1 gene corresponding to probes Nos. 6, 7, 8, 21 or 24.

According to a particular embodiment of this aspect of the invention, a method of inhibiting IL1R1 is provided. It is demonstrated that antisense inhibition at the level of the mature IL1RI mRNA transcript with exon-bridging antisense oligomers results in an efficient inhibition using lower amounts of oligomers and avoiding possible toxic effects to the treated cells. The present invention more particularly relates to the use of one or more of the exon-bridging antisense oligomers directed against IL1R1 described herein, for the inhibition of IL1R1 in chondrocytes, chondrocyte precursors, fibrochondrocytes, or fibroblasts, more particularly in osteoarthritic chondrocytes either in vitro or in vivo.

A particular embodiment of this aspect of the present invention further relates to a method of inhibiting excessive IL1 activity in conditions characterised by excessive signalling through the IL1/IL1R pathway in humans or animals. It is demonstrated that in such conditions, more particularly in osteoarthritis, inhibition of the receptor by the method of the invention has a number of advantages. Moreover, this overcomes the inherent disadvantages associated with ligand-inhibition.

Thus, according to one embodiment of the invention, exon-bridging probes are used as a medicament for the inhibition of expression of human IL1RI.

Accordingly, another aspect of the invention relates to a pharmaceutical composition comprising one or more exon-bridging antisense oligomers for the inhibition of the expression of a target gene and further comprising at least one pharmaceutically acceptable carrier. More particularly, the invention relates to pharmaceutical compositions comprising one or more of the IL1R1 inhibiting exon-bridging antisense oligomers described herein.

A particular embodiment of the pharmaceutical compositions of the present invention are compositions comprising one or more exon-bridging antisense oligomer against IL1R1. More particularly the composition comprises one or more IL1R1 exon-bridging antisense oligomers having a sequence of 15 to 30, more particularly 20 nucleotides, which are complementary to the sequence in the IL1R1 cDNA bridging exons 02-03 or 05-06. Most particularly, the composition comprises one or more IL1R1 exon-bridging antisense oligomers selected from the group consisting of probes Nos 6 (SEQ ID NO:6), 7 (SEQ ID NO:7), 8 (SEQ ID NO:8), 21 (SEQ ID NO:21) and 24 (SEQ ID NO:24) described herein and exon-bridging antisense oligomers having at least 70% sequence identity with the sequence of probe NO. 6, 7, 8, 21 or 24 described herein, more particularly exon-bridging antisense oligomers which additionally have at least 70% sequence identity with the complementary sequence of the cDNA of the IL1R1 gene corresponding to probes NO. 6, 7, 8, 21 or 24.

Another aspect of the invention relates to the use of the IL1R1 exon-bridging antisense oligomers of the present invention in the manufacture of a medicament for the treatment or prevention of a disease characterized by a cartilage or osteochondral defect, more particularly osteoarthritis.

Another aspect of the invention relates to the use of the IL1R1 exon-bridging antisense oligomers of the present invention in the manufacture of a medicament for the treatment or prevention of a disease selected from the group consisting of neuropathies, such as diabetic neuropathy, immune-mediated damage to the peripheral nervous system, heat hyperalgesia, Guillain-Barre syndrome, AIDS, bone disorders, such as osteoporosis caused by lymphomyeloid proliferative diseases, bone resorption, as occurring in a variety of diseases including osteoporosis, periodontal disease and rheumatoid arthritis, atheromatosis, coronary heart diseases, acute renal failure, asthma and nasal polyposis.

Another aspect of the invention relates to a method for producing an exon-bridging antisense oligomer for the inhibition of expression of a target gene comprising the steps of:

1) determining the exon-exon boundaries in the sequence of a spliced mRNA of the target gene,

2) selecting a sequence with a length between 15 and 30 residues bridging an exon-exon boundary in the spliced mRNA of the target gene, the sequence comprising

at its 5' end or 3' end at least 4 residues identical to a sequence 5' of said exon-exon boundary and,

optionally said sequence comprising at its 3' or 5' end a maximum of 11 residues identical to the sequence 3' adjacent of the targeted exon-exon boundary in the target mRNA.

3) producing an antisense oligomer which consists of a sequence which is has at least 70% sequence identity with a sequence complementary to the sequence selected in step 2.

According to a particular embodiment, the invention relates to a method as described above, wherein step 2 further comprises one or more of the steps selected from the group consisting of:

a) determining whether the GC content of the sequence determined under (2) is above 45 %,

b) determining whether the Tm of each of the sequences 3' and 5' of the exon-exon boundary within the sequence is below 32-36°C,

c) determining whether the oligomer has a sequence identity below 70% with mature mRNA other than the mature mRNA or DNA of the target gene;

And selecting the one or more sequences which fulfil the criteria of one or more of steps a to c.

The concept of the present invention, underlying the design and use of exon-bridging antisense oligomers can be applied to any gene of which the intron exon

structure is known or can be determined. Thus it applies to all eukaryotic genes, but more specifically is of interest for the modulation of mammalian and human genes. The production of multiple gene products from genes which have different splice variants can be inhibited with exon-bridging antisense oligomers directed against two adjacent exons which are common to all splice variants or with a combination of exon-bridging antisense oligomers which target all possible splice variants.

Moreover the concept of the invention can be applied for other methods of inhibiting gene expression based on mRNA inhibition, such as, but not limited to methods of RNA interference, short-hairpin RNA, etc. whereby these methods make use of probes targeting the exon-bridging regions of the mRNA. Most particularly the present invention provides for inhibition of IL1R1 based on using complementary sequences to the sequences spanning the coding exons in the cDNA of the IL1R1 gene.

Detailed description of the invention

Definitions:

The term **"gene"** refers to a DNA sequence comprising several operably linked DNA fragments such as a promoter, a 5' untranslated region (the 5'UTR), a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. The 5'UTR, the coding region and the 3'UTR are transcribed into a primary RNA transcript. Typically, the sequence of the gene comprises both introns (sequence of DNA that is initially transcribed into primary RNA but is cut out of the mature mRNA transcript) and exons (an exon being a sequence of DNA which together with other transcribed exons of the gene, by splicing of the primary transcript, results in the mature mRNA which comprises the coding sequence). The **'target gene'** as used herein refers to the gene of which the expression is targeted and from which the mature mRNA(s) originate (through a process of transcription and splicing) which are used to design the probe(s) of the invention (also referred to as 'target mature mRNA').

The term **"gene expression"** relates to the process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (e.g., transfer and ribosomal RNAs).

The term **"sequence identity"** between regions of mRNA or DNA means that, when two particular nucleotide sequences (RNA or DNA) are aligned, the percentage of nucleotides, i.e. the number of positions with identical nucleotides divided by the total number of nucleotides in the particular nucleotide sequence, is higher than 70%, particularly at least 80%, even more particularly at least 90%, most particularly at least 95-99%, most particularly is 100%.

The terms "**complementary**" or "**complementarity**" refer to base pairing between A and T(orU) and between G and C bases in a nucleotide double strand. For example an oligonucleotide with sequence 5'-GGACTT-3' is complementary to an oligonucleotide with sequence 5'-AAGTCC-3'. Complementarity can be also expressed in percentages as the ratio of the number of base pairs where perfect base pairing occurs over the total number of position where base pairing can occur. Within the context of this invention, an oligomer is complementary with the target mature mRNA when base paring occurs for at least 70%, preferably for at least 80%, even more preferably at for at least 90%, most preferably at for least 95-99%, more specifically for 100% of the oligonucleotide sequence. An oligonucleotide is 100 % complementary when A T(or U) and G C base pairing with the target mature mRNA sequence occurs at every base pair of the oligonucleotide.

The term **"primary RNA transcript"** refers to RNA as transcribed from the gene but which has not been spliced yet into mature mRNA.

The term **"mature mRNA"**, **"processed mRNA"** or **"spliced mRNA"** refers to mRNA which has been spliced from the primary RNA transcript

The term **"exon-exon boundary"** refers to the link between two bases in a mature mRNA sequence corresponding to the connection of the 3' end of an exon and the 5' end exon of another exon of the genomic sequence, more particularly the sequence corresponding to the connection between two coding exons. The 5'end

of an exon-exon boundary refers to the sequence 5' and adjacent to the exon-exon boundary which corresponds to the 3' end of an exon in the genomic DNA. The 3' end of the exon-exon boundary refers to the sequence 3' and adjacent to the exon-exon boundary which corresponds to a sequence at the 5' end of the next exon which has been spliced into the mature mRNA.

The term **"oligomer"** refers to a plurality of nucleosides joined together via covalent linkages in a specific sequence from naturally and/or non-naturally occurring nucleobases, and includes oligonucleotides as well as oligonucleosides. The term **"oligonucleotide"** refers to an oligomer comprising a plurality of nucleosides joined together by phosphorous linkages. The term **"oligonucleoside"** refers to oligomers comprising a plurality of nucleosides joined together by non-phosphorous linkages. The oligomers of the present invention can also comprise different modifications of the side chains and covalent linkages. Possible modifications are discussed in detail, for example, in US patent application 20030148969.

The term **"effective amount"** in the present invention refers the concentration of an antisense oligomer which can modify gene expression, more particularly decrease gene expression, by inhibition of the target mRNA function. An amount is said to be effective when it results in a decreased expression of the gene with at least 30% preferably at least 50%, more preferably at least 70% even more preferably at least 90 %, and most preferably at least 95 %. Expression can be measured for example by comparing the protein concentration of an expressed target gene in untreated cells compared with cells being treated with an antisense oligomer. In a particular embodiment the protein concentration of an expressed target gene is determined by measuring the fluorescence as MFI after binding of an antibody to the expressed protein. This is described in detail in the experimental part of this application.

Alternatively an effective amount of an antisense oligomer is an amount that modifies (increases or decreases) the expression of a gene downstream of the targeted gene. Effective amounts are those amounts that lead to a modification in expression of gene downstream of the targeted gene, by at least 15 %, preferably at least 30 % and more preferably at least 40%. In a particular embodiment of the

invention, the effect of an antisense oligomer directed against IL1RI expression is measured by the increase in expression of genes encoding one or more ECM compounds, such as aggrecan.

Expression level of a gene can also be measured for example by comparing the mRNA concentration of a target gene in untreated cells versus cells being treated with an antisense oligomer. This can be done for example by Northern analysis.

The present invention relates to the use of oligomers for the modulation of gene expression. More particularly, the expression of one or more genes is modulated by specific hybridization of the oligomers of the invention with one or more target nucleic acids, more specifically one or more target mature mRNAs, interfering with the function of the target mature mRNA. Functions of the target mature mRNA primarily relate to translation of protein from the mature mRNA but moreover include translocation of the mature mRNA and any other effects facilitated by the mature mRNA.

In order to solve the problem of specifically and efficiently targetting mature mRNA, the present invention provides the use of exon-bridging antisense oligomers or oligonucleotides. The term **"exon-bridging antisense oligomer"** refers to an antisense oligomer consisting of a 5' and a 3' end, whereby the 5' end is complementary to the 5' end of an exon of a gene and the 3' end is complementary to the 3' end of the preceding exon of that gene in the mature mRNA. An exon-bridging oligomer is therefore complementary to the mature mRNA. Because the sequence of the 5' and 3' end of an exon-bridging oligomer are separated in the genomic DNA sequence and primary RNA transcript by (at least) an intronic sequence, exon-bridging oligomers are not complementary to genomic DNA or primary RNA. The concept of an exon-bridging oligomer is exemplified for the different exon-bridging sequences of human IL1RI in Table 3.

The term exon-bridging is not to be confused with the term exon-spanning probes used in the literature, which are longer polynucleotides complementary to genomic DNA and comprising a sequence corresponding to (or complementary to) at least one exon and adjacent intronic sequences.

The present invention relates to the use of exon-bridging oligomers for *in vivo* or *in vitro* modulation of gene expression. The antisense oligomers used in accordance with this invention can be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is commercially available, such as, but not limited to for example, the equipment from Applied Biosystems.

According to the present invention, the exon-bridging antisense oligomers used for the specific inhibition of mature mRNA have a length of between 13 and 30 nucleotides and even more preferred between 15 and 22 nucleotides. Particularly suitable according to the present invention are oligomers of 20 nucleotides. The minimal total oligomer strand length to ensure, on a statistical basis, specificity towards a certain mRNA has been calculated to be 13 bases (1). Optionally, oligomers can have a length which exceeds 30 nucleotides when, apart from the exon-bridging sequence, insertions such as internal hairpins or 5' or 3' extending parts, eg for use as a tag, are present The choice for a particular length of an oligomer can be influenced by considerations such as Tm and GC content.

In a particular embodiment, the oligomer of present invention has a sequence which is at least 70%, preferably at least 80%, more preferably at least 90% and most preferably at least 95% identical to a sequence complementary to the target mature mRNA .

In another particular embodiment the sequence of the antisense exon-bridging oligomer (or their complementary sequence) shows as little sequence identity as possible with any other part of the genome. Possible interference with unrelated (encoding other mRNAs than the target mature mRNA) genomic DNA or mRNA is minimised or avoided as assessed by NCBI BLAST^R (3-7). Possible interference with mRNA other than the target mRNA is avoided by using those oligomers which complementary sequence is not more than 70 % identical, preferably not more than 60 % identical, more preferably not more than 50 % identical and most

preferably not more than 30 % identical to a sequence other than the target mRNA sequence. Similarly, interference with genomic DNA is avoided by using those oligomers with a sequence or a complementary sequence which is less than 70 % identical, preferably less than 60% identical, more preferably less than 50% identical and most preferably less than 30% identical to a genomic sequence, including regions representing genes (intron, exons, 5' and 3' regulatory sequences), and regions which do not encode genes, so called junk DNA.

In another particular embodiment the antisense oligomers of the invention have a sequence which is complementary to an exon-exon boundary within the 5' half of the spliced mRNA of a gene. Exon-bridging antisense oligomers directed against mRNA sequences corresponding to the first half of the spliced mRNA will more efficiently silence the target gene than those corresponding to the last third.

In another particular embodiment the exon-bridging antisense oligomers of the present invention do not comprise a sequence of more than 11 consecutive nucleotides which are complementary to the 3' or 5' end of the exon-exon boundary in the target mature mRNA.

Additionally or alternatively the oligomers of the present invention have a GC content of at least 45%. Since in general, genes are located on GC rich regions of the genome and merely 20% of the genes have a GC content of more than 45% (Lander et al., 2001, *Nature* **409**:860-921), a higher GC content restricts the chances of non-specific binding to genomic DNA.

Additionally or alternatively the exon-bridging probes can be designed so that the stretches of nucleotides complementary to the 5' and 3' end of the exon-exond boundary in the target mRNA will not anneal to 100% complementary DNA stretches. This can be achieved by ensuring that the 5' and 3' end of the probe have a Tm (melting temperature) of about 32-37° C, depending on the temperature at which inhibition is desired. This Tm provides an unstable annealing at the human body temperature (adjustments can be made for different parts of the body)

or the temperature at which in vitro inhibition in somatic cells is performed. The Tm for short nucleotides is defined by the length and the GC content of the probe (Wallace RB et al., 1979, *Nucleic Acids Res* 6:3543-57). Thus, according to one embodiment of the invention the length of the probes is adjusted to ensure a Tm of less than 32-37°C. Alternatively, for a particular length, the target sequence of the mature mRNA can be selected so as to ensure an appropriate GC content.

Thus, for a sequence complementary to the 5' or 3' end of the exon-exon boundary having a length of 11 nucleotides, the GC content should not be more than 45% (5/11) - 64% (7/11). For a length of 10 nucleotides, the GC content should preferably be no more than 60-80%. Particularly, the length of the exon-bridging probes can be designed so that the 5' and 3' end are limited to 8 nucleotides or less. For these lengths a GC content of up to 100%, will anneal to 100% complementary sense DNA stretches with Tm of 32°C.

Additionally or alternatively, the oligomers of the present invention can be designed to ensure that they are devoid of GGG sequences or longer G repeats in order to avoid self-interaction that would lead to self-composed complexes within oligomers, which no longer hybridize to the target mRNA.

In another embodiment the oligomers of the present invention are stabilised by a modification such as phosphorothioate. Other types of stabilizing modifications as described in e.g. US application 20030148969 are envisaged within the context of the present invention, provided that these do not affect the ability of the probes to enter and leave the cells.

The present invention relates to the use of exon-bridging probes for the *in vitro* or *in vivo* modulation of gene expression, by interfering with mature mRNA function. The use of exon-bridging oligomers ensures a limited binding of the oligomers to genomic DNA, which avoids certain drawbacks of classical antisense oligomers. Local binding of an antisense oligomer to the genome of the target gene can result in local activation or inactivation of neighbouring genes. This can interfere with the

desired inhibition or this can also lead to toxicity and death of cells being treated with the antisense oligomer. Moreover, the present invention describes methods of further reducing interference of exon-bridging probes with genomic DNA.

Thus, in a particular embodiment of the invention exon-bridging antisense oligomers are used of which the complementary sequence has no more than 70% sequence identity with genomic DNA other than the DNA encoding the target mRNA. Preferably, the oligomers are used of which the complementary sequence has less than 85% sequence identity with any genomic DNA sequence. Additionaly or alternatively the sequence of the oligomer is designed such that the oligomer contains a sequence of at most 11 consecutive residues which is identical to either sequence 5' or 3' of the exon-exon boundary. Such an oligomer has no more than 11 residues complementary to the exon in the genomic sequence and is unlikely to bind to the genomic DNA corresponding to the target mature mRNA. Additionally or alternatively, the method of the invention is performed using exon-bridging probes of which the sequences complementary to the 5' and 3' end of the exon-exon boundary of the target mRNA have a Tm of less than 32-37°C, depending on the target tissue or on the inhibition conditions. Additionally or alternatively, by performing the *in vitro* or *in vivo* method with exon-bridging oligomers which have a GC content above 45 %, preferably above 60%, non specific hybridisation with non coding parts of the genome (which are poor in GC content) is minimised.

Specific binding to the target mature or spliced mRNA is achieved by using an exon-bridging oligomer of which complementary sequence has at least 70% sequence identity with the target mature mRNA.

According to another aspect of the invention, a method of antisense inhibition is provided which requires a limited amount of antisense probe. Due to the reduced binding of exon-bridging oligomers to genomic DNA, less antisense is scavenged and the method can be performed with a limited amount of oligomer. More particularly, *in vitro* inhibition can be obtained with a concentration of an oligomer in the cell culture medium of less than 100 nM, preferably between 1 and 10 nM

and most preferably about 1nM or less. Also for *in vivo* inhibition, a lower amount of one or more exon-bridging oligomers can be administered compared to non exon-bridging oligomers of the prior art in order to achieve the same effect.

In a particular embodiment of the invention, the *in vitro* or *in vivo* modulation of gene expression using exon-bridging oligomers which inhibit mature mRNA function, is reversible. Reversal of a biological exon-bridging oligomer effect can be achieved through changes in the environment of the target cells. *In vitro*, mammalian cells can survive and be metabolically active at temperatures between 30 and 38°C. For a given Tm value of a probe, increasing or decreasing the environmental temperature above or below that value will affect annealing of the probe to RNA/DNA. The temperature within different parts of the body can also vary. Intra-articular temperatures can vary in between 32 and 38°C (Oosterveld et al., 1992, *Arthritis Rheum.* **35**, 146-51; Oosterveld & Raster, 1994, *Arthritis Rheum.* **37,** 1578-82). The inhbitory effects of an exon-bridging oligomer can be influenced by heating or cooling of the body part to a temperature above or below the Tm of the total probe (which is according to standard practice higher than that of the body part where inhibition is desired).

According to a particular embodiment of the *in vitro* method of inhibiting the expression of a target gene with exon-bridging oligomers, the cells are contacted with the probes in the absence of DNA tranfecting agents. DNA transfecting agents are often toxic and will reduce the number of living cells in a population which is treated with antisense oligomers. The absence of DNA transfecting agents is also desirable for *in vivo* applications. Nevertheless, it is envisaged within the context of the present invention that transfecting agents or other additives can be used (e.g. such as those described in US application 20030148969).

A particular aspect of the invention relates to the use of exon-bridging probes for the *in vivo* or *in vitro* modulation of gene expression by inhibition of all mature mRNAs of a target gene. Target genes giving rise to more than one mature mRNA include genes where alternative splicing results in splice variants. According to this

aspect of the invention translation of the mature mRNA of the different splice variants is inhibited. Inhibition of the translation of all splice variants is achieved when the targeted exon-exon boundary is common for all splice variants or when several exon-bridging oligomers are used to cover all possible splice variants.

According to another aspect, the invention relates to the use of multiple probes for the modulation of gene expression. According to this aspect a combination of different exon-bridging probes, each directed to a different exon-exon boundary in the mature mRNA sequence is used to ensure inhibition of the mature mRNA.

The *in vitro* or *in vivo* method of inhibiting the expression of a target gene with exon-bridging oligomers is applicable to a wide variety of cells including human and non-human mammalian cells, including expanding cells or cells which do not divide, such as confluent cell cultures. Cells can be freshly isolated cells from a tissue or can be expanded or passaged cells. In a particular embodiment the *in vitro* method is performed on connective tissue cells, preferably osteochondral cells, more preferably mature chondrocytes and chondrocyte precursors. Cells can be healthy cells or in a particular embodiment osteoarthritic cells.

Another aspect of the invention relates to a pharmaceutical composition comprising one or more exon-bridging antisense oligomers with a length between 15 and 30 residues for the inhibition of the expression of a target gene and further comprising at least one pharmaceutically acceptable carrier. Carriers and other additives which are compatible for a pharmaceutical composition comprising antisense oligomers are described in detail in US application US20030148969.

Another aspect of the invention relates to a method for producing an exon-bridging antisense oligomer for the modulation of expression of a target gene by inhibition of the corresponding mature mRNA function, wherein the following steps are performed: 1) determining the exon-exon boundaries in the sequence of one or more mature mRNA of said target gene; 2) selecting a sequence with a length between 15 and 30 residues bridging an exon-exon boundary in the spliced mRNA

of said target gene, such a sequence comprising at its 5' or 3' end at least 4 residues identical to a sequence 5' of said exon-exon boundary and, optionally said sequence comprising at its 3' or 5' end a maximum of 11 residues identical to the sequence 3' adjacent of said exon-exon boundary; 3) producing an antisense oligomer which consists of a sequence which is at least 70% complementary to the sequence selected in step 2.

According to further embodiments of the invention, the method for producing an exon-bridging oligomer can further comprise one or more of the following steps: a) determining whether the GC content of sequence identified in step 1 is above 45 % b) determining whether the Tm of a sequence complementary to then sequence identified in step 1 is above 37°C, c) determining whether the sequence complementary to the sequence identified in step 1 has a sequence identity of less than 70% with any mature mRNA other than the mature mRNA of the target gene and d) determining whether the Tm of the sequences 5' and 3' end of the exon-exon boundary in the sequence identified in step 1 is below 32-36°C. Oligomers are then produced which correspond to one or more of the criteria determined in steps a) to d) following steps 2 and 3 described above.

Another aspect of the invention relates to the use of an exon-bridging antisense oligomer with a length between 15 and 30 residues for depressing cytokine pathways *in vivo* or *in vivo* through the downmodulation or silencing of the appropriate receptors. Such an inhibition of modulation can have important therapeutic consequences in conditions of which the pathogenesis and the progression are dependent of the overexpression of these cytokine pathways. In this context, inhibition of the signals of pathways such as, but not limited to the TNF/TNFR, the IL2/IL2R, the IL11/IL11R, the IL14/IL14R, the IL15/IL15R, are envisaged.

Alternatively, the method of the invention can be used for the silencing or modulation of other cell functions, which have a detrimental effect causing

pathological conditions. Examples include the (over)production of metalloproteinases in tissue destruction, COX-2 in inflammation, ...etc.

A particular aspect of the invention relates to the use of an exon-bridging antisense oligomer according to the present invention for the *in vivo* or *in vivo* inhibition of the expression of Interleukin 1 Receptor Type I (referred to as IL1RI).

In a preferred embodiment of the invention IL1RI is human IL1RI. Human IL1RI (hIL1RI) is documented in OMIM entry 147810. The hIL1RI mRNA sequence is accessible via Genbank (NM_000877 or M27492) In these entries the coding sequence for ILR1 starts at nucleotide 83 and ends at nucleotide 1792. The genomic sequence of hIL1RI is presented in Genbank Accession AF531102. Herein the exons are located at nucleotides:

1296-1371 (non coding "exon 0"); 5079-5139 (exon 1); 12129-12363 (exon 2); 12470-12659 (exon 3); 13468-13636 (exon 4); 15953-16018 (exon 5); 19156-19273 (exon 6); 20042-20193 (exon 7); 21942-22085 (exon 8); 22835-23002 (exon 9), 23710-27231 (exon 10) (exon 10 contains coding sequence from 23710 to 24116) (exon 10).

The sequences bridging the above-cited exons in the mRNA of the IL1R1 are the target of the probes of the present invention. Particularly suited in the context of the present invention are the sequence bridging exons 02-03 and the sequence bridging exons 05-06.

Complete or partial cDNA or genomic IL1RI sequences for non-human mammals are determined for e.g. mouse (NM_008362), rat (NM_013123), horse (AB020338) or can be cloned with standard molecular biology techniques, for example with PCR primers designed for conserved regions of the IL1RI receptor.

Oligomers which have been designed for *in vitro* or *in vivo* inhibition of the expression human IL1RI according to the present invention are presented in table 3 and in the examples.

Exon-bridging oligomers for IL1RI from other animals or for any other gene can be designed when the mRNA sequence around the exon-exon boundaries is known.

According to a particular embodiment of the present invention, the sequences bridging the exons of the IL1R1 gene identified within the context of the present

invention can be used for the design of complementary probes which are not classical antisense probe but other inhibitory probes such as short-hairpin RNA or dsRNA. The main requirement for these probes according to the present invention is that it is a sequence with a length between 15 and 30 residues and that it comprises at its 5' or 3' end at least 4 residues identical to a sequence 5' of said exon-exon boundary.

The modulation of IL1RI expression with exon-bridging antisense oligomers can be used for influencing *in vivo* or *in vitro*, the metabolism of extracellular matrix compounds such as type II collagen , aggrecan and fibronectin. Thus, in one aspect the invention relates to the use of exon-bridging antisense oligomers for the treatment or prevention of a disorder caused or manifested by an excessive IL1RI activation or expression.

A particular embodiment of the invention relates to the use of the exon-bridging probes of the invention in the treatment or prevention of a cartilage or osteochondral defect, a defect of joint related tissues and defects of other tissues of a fibrocartilage nature such as intervertebral discs, as well as in the formation of bone or cartilage in other indications, e.g. cosmetic surgery.

According to a particular embodiment, the present invention relates to the modulation of IL1RI in order to control the excessive Interleukin-1 (IL1) signalling via Interleukin-1 Receptor type I (IL1RI) in chondrocytes which plays an important role in osteoarthritis (OA). Based on the determination of the different factors regulating the production of ECM in healthy and osteoarthritic chondrocytes, it was established that the IL1/IL1R pathway is the most promising target for therapeutic activity. Increasing growth factor activity would make no sense as the IGF-1/IGF1R pathway is already stimulated in OA tissue, and does not result in an accumulation of ECM compounds in the cell associated matrix (CAM) while newly synthesised ECM products are readily degraded in the presence of an activated IGF/IGF1R pathway. It can be anticipated that the inhibition of IL1/IL1RI pathway by downregulating IL1 levels would be only partially successful when the

upregulation of the CAM is desired. Remaining levels of IL1 would still result in an unacceptable amount of receptor activation. It is demonstrated that using the oligomers of the present invention directed against mature mRNA of IL1RII, production of ECM compounds, more particularly aggrecan is completely restored. The low price and fast and simple manufacture of oligonucleotides are important advantages of antisense technology compared to the time and efforts needed for the development of small inhibitory molecules, inhibitory antibodies or inhibitory soluble receptors.

According to the present invention, excessive IL1/IL1RI pathway activity can efficiently be inhibited by the use of exon-spanning probes directed against IL1RI mature mRNA. Moreover, the use of the exon-spanning probes of the invention minimizes the risk of interaction with genomic DNA which could negatively affect other cellular processes.

Thus, one aspect of the invention relates to the use of an exon-bridging antisense oligomer with a length between 15 and 30 residues for the manufacture of a medicament for the treatment or prevention of a disorder caused or manifested by an excessive IL1RI expression. More particularly, the present invention relates to the use of exon-bridging probes for the inhibition of IL1RI expression in osteoarthritis. However, other applications of the IL1RI-directed probes of the present invention include (but are not limited to) neuropathies such as diabetic neuropathy, immune-mediated damage to the peripheral nervous system, heat hyperalgesia, Guillain-Barre syndrome, AIDS, bone disorders such as osteoporosis caused by lymphomyeloid proliferative diseases, or to bone resorption in a variety of diseases including osteoporosis, periodontal disease and rheumatoid arthritis. The IL1/IL1RI pathway has furthermore been implicated in atheromatosis, in coronary heart diseases, in some forms of acute renal failure, in asthma and in nasal polyposis, and in a variety of disorders in humans.

Moreover, the application of IL1RI inhibition applies in all *in vitro* an *in vivo* experimental models for the study of the above-mentioned diseases.

Another aspect of the invention relates to the use of an exon-bridging antisense oligomer according to the present invention as a medicament.

Another aspect of the present invention relates to the use of an exon-bridging antisense oligomer according to the present invention for the production of a medicament for the treatment of any one of the diseases mentioned above.

Brief description of the figures

The following Examples, not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Figure 1: Passage of different base-length oligonucleotide probes (10, 20 and 30-mer poly A strands) through intact chondrocyte. Upper panel: Ability of fluorescent probes to enter the cytoplasm. Ordinate: mean fluorescence intensities of the cells as measured by FACS. Abscis: incubation time of the cells with the probes; The results show that probes shorter than 30 bases freely enter chondrocytes at 37°C; Lower panel: Remnant Fluorescence upon repeated washing at 37°C. Ordinate: mean fluorescence intensities of the cells as measured by FACS. Abscis: number of cell washes. From the lower panel it can be deduced that free probes were completely washed away by two washings at 37°C.

Figure 2: Annealing of FITC-labeled antisense DNA probes (FITC-probes) to mRNA in intact chondrocytes in function of exposure time. Ordinate: mean fluorescence intensity of the cells after incubation with the FITC-probes as measured by FACS. Abscis: incubation time (hours) of the cells with the FITC probes. A two-hour exposure period to the probes was enough to ensure maximal annealing to strands of specific mRNA, as shown with an aggrecan mRNA-specific exon-spanning antisense DNA probe in phenotypically stable chondrocytes.

Figure 3: Chondrocyte populations obtained from 3 different donors incubated with 0 (= control, polyA probe), 1, 2, 3 or 4 exon-bridging FITC-probes directed to aggrecan. Dots represent mean values of triplicate experiments. Ordinate: Chondrocyte MFI; Abscissa: number of probes used. The MFI values of the 3 chondrocyte populations linearly increase with the number of aggrecan mRNA directed probes used.

Figure 4: Example of an exon-bridging antisense oligonucleotide (5'-AAGATGAATTTCTTACCACGCA-3') (SEQ ID NO: 384) complementary to the sequence bridging exons 2 and 3 in the mature mRNA of the IL1R1 gene.

Figure 5: Illustration of exon-bridging probes as compared to exon-flanking probes.

EXAMPLES

Methodology

Isolation of chondrocytes

Human articular chondrocytes were isolated from articular cartilage obtained at autopsy within 24 hours post mortem. Knee joints showing focal OA lesions were used to obtain visually intact cartilage and OA cartilage. The cartilage tissue was sampled from the femur condyles, diced in small fragments and digested in a spinner bottle with a series of enzymatic solutions in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) with 0.002M/ml L-glutamine and antibiotics and antimycotics (GIBCO BRL) (9,10). Cartilage was first treated with 0.25% (w/v) of sheep testes hyaluronidase (Sigma, St. Louis, MO, USA) for 120 min and 0.25% of Pronase (Streptomyces griseus Pronase E; Sigma) for 90 min at 37°C. After an overnight period in DMEM supplemented with 10% foetal calf serum (FCS, GIBCO BRL), a 3 to 6 hours' incubation with 0.25% collagenase (Clostridium histolyticum; Sigma) in DMEM containing 10% FCS at 37°C resulted in the liberation of isolated cartilage cells. Depending on the condition of the tissue,

50-150x10^6 chondrocytes could be obtained from femoral condyles of one individual and over 95% of the cells were viable (Trypan Blue exclusion test) after isolation.

Culture of chondrocytes in alginate gel

Chondrocyte cultures in alginate beads were prepared as described elsewhere (Guo. et al., 1989, *Conn Tiss Res* 9: 277-97) with some modifications. Chondrocytes suspended in 1 vol. double-concentrated Hanks' Balanced Salts Solution without calcium and magnesium (HBSS; Gibco) were carefully mixed with an equal volume of 4% alginate (low-viscosity alginate from Macrocystis pyrifera; Sigma) in HBSS, autoclaved for 15 min. The final cell concentration was 5 x 10^6 chondrocytes per ml in 2% alginate. The chondrocyte/alginate suspension was then slowly dripped through a 23-gauge needle into a 102 mM calcium chloride solution. The beads were allowed to polymerize for 10 min. at room temperature. The calcium chloride was then removed, the beads were washed three times with 0.15 M sodium chloride and finally maintained in a 6-well plate in 4 ml of Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) with 10% fetal calf serum (FCS, GIBCO BRL) and 50 µg ascorbate per ml in an incubator at 37°C under 5% CO_2. The chondrocyte cultures consisted of 1 x 10^6 cells per culture. The nutrient medium was replaced twice weekly for 7-14 days.

Flow cytometry detection

Preparation of chondrocytes for flow cytometry (12): After the respective culture periods, the medium of the cultures was aspirated and the alginate beads were washed and dissolved by incubation for 10 min. with 3 ml of 55 mM tri-sodium citrate dihydrate pH 6.8, 0.15 M NaCl at 25 °C. The resulting suspension was centrifuged at 1500 rpm for 10 min. to separate cells with their cell-associated matrix (CAM) from the constituents of the interterritorial matrix. Aggrecan in the CAM were tested directly after incubation with the appropriate antibody for 30 min in the dark at 4°C. 20µl of 50µg/ml antibody were used to react with 2x10^5 cells resuspended in 100µl PBS.

Antibodies used for flow cytometry: Mouse anti-human chondrocyte-specific aggrecan monoclonal antibody (mAb) (clone 4D11-2A9, Biosource Europe, Nivelles, Belgium; subclass: IgG_1) was shown to react specifically with the G1-domain of the invariable hyaluronan-binding region of the human aggrecan protein core molecule, and was used to detect the aggrecan in the chondrocyte CAM. The anti-aggrecan mAb was conjugated with Phycoerythrin (PE, Sigma-Aldrich) as described (Kronick et al., 1983, *Clin Chem* 29: 1582-6). The conjugated mAb was used in a direct immunofluorescent staining protocol for flow cytometry. Mouse anti-human mAb (subclass: IgG_1) against IL1RI were obtained from R&D systems (Abingdon, United Kingdom; clone 35730.111) and labelled with FITC using a standard protocol (Wood BT et al, 1965, *J Immunol* 95: 225-9.). Appropriate FITC- and PE-labeled isotype matched mouse IgG_1 were used as negative controls.

Flow cytometric analysis: Stained cells were analysed on a flow cytometer (FACSort, Becton Dickinson, San Jose, CA, USA) with CELLQuest software. From each sample, 15,000 events were analysed. Cells were gated on forward and side scatter to exclude dead cells, debris and aggregates. Propidium iodide was additionally used to exclude dead cells when CAM aggrecan was analysed (Wang L et al., 2001, Osteoarthritis Cartil. 9:73-84). The mean fluorescence intensity (MFI) of the positive cell population, which is due to the binding of the conjugated antibodies to the specific antigen, was used to quantify the presence of aggrecan in the CAM or to assess the expression of IL1RI on the plasma membrane. MFI values were obtained by subtraction of the MFI of the negative control population from the MFI of the positive stained population. For comparison between experiments Quantum Simply Cellular Microbead Kit (Sigma) was used to calibrate the fluorescence scale of the flow cytometer (Wang L et al., 2001, *Osteoarthritis Cartil.* 9: 454-62). The microbeads were stained and processed in parallel with the cell samples using the same amount of FITC-labeled antibodies and incubation time. The fluorescence scale of the cytometer was adapted before every experiment in order to keep identical MFIs for the four peaks of the calibration beads. The MFI of cell samples was then analysed without changing any

instrument settings. The reproducibility and reliability of whole procedure has been demonstrated previously (Wang L et al., 2001, *Osteoarthritis Cartil.* 9:454-62).

Probes

The following probes were used:

negative control probes:

probe 1: PolyA 5'FITC-aaa.aaa.aaa.aaa.aaa-3' (SEQ ID NO: 1)

specific probes:

probe 2: aggrecan (antisense) 5'FITC-cacgatgcctttcac-3' (SEQ ID NO: 2)

probe 3: aggrecan (antisense) 5'FITC-aaagacctcaccctc-3' (SEQ ID NO: 3)

probe 4: aggrecan (antisense) 5'FITC-ggtatctgacggtct-3' (SEQ ID NO: 4)

probe 5: aggrecan (antisense) 5'FITC-ggcattgttgttgac-3' (SEQ ID NO: 5)

probe 6: IL1RI (antisense) 5'-gaattccttaccacg-3' (SEQ ID NO: 6)

probe 7: IL1RI (antisense) 5'-gatgaattccttaccacgca-3' (SEQ ID NO: 7)

probe 8: IL1RI (antisense) 5'-taagatgaattccttaccacgcaa-3' (SEQ ID NO:8)

Example 1: determination of the concentration of different factors involved in ECM production by chondrocytes.

Osteoarthritic (OA) cartilage is characterised by destructive processes. In order to identify the most appropriate target for inhibiting this destructive process, the expression of different factors influencing ECM production were compared in chondrocytes from healthy and degenerative tissues from the same joint (Table 1). The OA chondrocytes were found to show increased catabolic activity: IL1 alpha and beta concentrations inside cells were found to be increased and so were the plasma membrane levels of the signal transmitting IL1RI. However, the IL1 levels are so dramatically increased that activity of IL1 appears to be limited by the paucity of its signalling receptor IL1RI. Numbers of IL1 ligands in osteoarthritic chondrocytes were observed to overpower the presence of their receptors with a factor 20-30.

Increased activity of the IGF-1/IGF1R growth factor pathway illustrates a cellular attempt of repair. However, significantly impaired accumulation of ECM compounds (aggrecan (AGGR), type II collagen (COLII), fibronectin (FNECT) are indicative of the degradation in the diseased OA cartilage tissue which willl ultimately lead to severe disabling articular pathology. The IL1RII decoy receptor levels, which were found to be decreased, are apparently unable to control the increased activity of the auto/paracrine IL1/IL1RI pathway in OA chondrocytes.

Thus, in disease conditions such as osteoarthritis even a 50% or more drop of the IL1 concentrations in the extracellular environment would not make any difference as the activity of the IL1/IL1R pathway is regulated by a critical number of specific IL1 plasma membrane receptors. Based hereon it is concluded that inhibition of IL1R is the most appropriate target for inhibiting the excessive ECM destruction in OA and similar diseases.

Table 1: Homeostasis of the ECM by chondrocytes from normal and osteo- arthritic cartilage

	Normal mean ± 1SD	Osteo-arthritic mean ± 1SD	Difference	p-value
IL1α	21.50 ± 9.81	24.50 ± 9.93	+14 %	0.0013
IL1β	38.70 ± 0.80	51.70 ± 23.80	+34 %	0.0107
IL1RI	0.86 ± 0.48	1.61 ± 0.94	+87 %	0.0093
IL1RII	2.33 ± 0.83	1.66 ± 0.58	-28 %	0.0154
IGF1R	1.95 ± 1.18	3.23 ± 2.16	+66 %	0.0038
IGF-1	41.30 ±32.30	57.10 ± 42.40	+38 %	0.0099
COLLII	4.80 ± 2.79	3.34 ± 2.47	-30 %	0.0141
AGGR	15.50 ± 9.13	9.50 ± 5.89	-39 %	0.0461
FNECT	324.50 ±189.9	245.30 ±151.6	-24 %	0.0062

Example 2: Feasibility of using the exon-bridging probes.

The *in vitro* modulation of aggrecan accumulation in the cell-associated matrix (CAM) of human chondrocytes obtained from normal and osteoarthritic (OA) fibrillated articular cartilage was compared in the presence or absence of exon-bridging antisense oligomers targeting IL1RI expression.

In order to determine the requirements of the oligonucleotide probes for use in living cells, the physical factors, which influence cell-membrane permeability of probes were first evaluated. Human articular chondrocytes were obtained from three donors and the ability of 10-, 20- and 30-oligomer FITC-polyA probes, to enter the cytoplasm was tested. The same probes were used to find out if unbound FITC-probes could be washed out from the cells. Chondrocytes were isolated from alginate beads after 1 week of culture. 20 µl of 0.03mM of the probes in phosphate buffered saline (PBS) were added to 1.0 ml of DMEM containing 1.0×10^5 intact chondrocytes. To measure the optimum time for the probes to reach an equilibrium between the intra- and extracellular domains, the cells were kept in the dark at 37°C and every hour 300 µl of the cell suspension was analysed by flow cytometry.

From these experiments it appeared that probes shorter than 30 bases freely entered, and were washed away from chondrocytes at 37°C, in case they did not find their mRNA target (Figure 1).

Furthermore, time course experiments indicated that an incubation of two hours is sufficient for the probes to reach the highest intracellular concentration and to anneal optimally with the target mRNA. The results obtained after incubating phenotypically stable chondrocytes with an FITC-labelled aggrecan mRNA-specific exon-bridging antisense DNA probe (5'FITC-cacgatgcctttcac-3' - SEQ ID NO : 2) are provided in Figure 2; location of the aggrecan probe in the mRNA sequence of human aggrecan 1 isoform 2 precursor (Genbank accession number 013227) : 508-522.

To find out the optimal washout procedure for uncombined or negative control poly A probes, chondrocytes were incubated with the probes for two hours and were subsequently centrifuged up to 4 times at 1,500 rpm, during 10 minutes at 37°. The residual fluorescence intensity of the stained cells was then assayed by flow cytometry.

The poly A control probes were not able to stain the target cells as they did not combine with RNA or genomic DNA. Probes directed to chondrocyte-specific mRNA, i.e. specifically directed against aggrecan, on the other hand annealed with their targets and allowed the targetted mRNA to be detected by flow cytometry. The use of multiple probes linearly increased the mean fluorescence intensity of the stained cell population (Figure 3).

Example 3: design of IL1RI exon-bridging antisense oligonucleotides

Table 2 demonstrates parts of the hIL1RI cDNA sequence which can be used for the design of exon-bridging oligonucleotides and the corresponding antisense sequence.

Table 2a : hIL1RI exon-bridging sequences

exon		SEQ ID NO	location in Genbank Accession NM_000877
0-1	5′-ctccaagaagaat-atgaaagtgttac-3′	9	70-95
1-2	5′-ttctctggaggctg-ataaatgcaagg-3′	10	130-155
2-3	5′-ttgcgtggtaag-aaattcatcttact-3′	11	367-392
3-4	5′-acagtggtataag-gattgcaaacctc-3′	12	556-581
4-5	5′-atttattactctag-aggaaaacaaac-3′	13	724-749
5-6	5′-ggaagtagacttgg-gatcccagatac-3′	14	790-815
6-7	5′-agactattacag-tgtggaaaatcctg-3′	15	910-935

7-8	5′-gttaatatatccag-tcactaatttcc-3′	16	1060-1085
8-9	5′-tctcccaataaaag-cttcagatggaa-3′	17	1204-1229
9-10	5′-ctacgttggggaag-acattgttgagg-3′	18	1372-1397

Table 2b: Exon-bridging antisense sequences of hIL1RI.

exon	Exon-bridging antisense	SEQ ID
0-1	5′-gtaacactttcat-attcttcttggag-3′	19
1-2	5′-ccttgcatttat-cagcctccagagaa-3′	385
2-3	5′-agtaagatgaattt-cttaccacgcaa-3′	386
3-4	5′-gaggtttgcaatc-cttataccactgt-3′	387
4-5	5′-gtttgttttcct-ctagagtaataaat-3′	388
5-6	5′-gtatctgggatc-ccaagtctacttcc-3′	389
6-7	5′-caggattttccaca-ctgtaatagtct-3′	390
7-8	5′-ggaaattagtga-ctggatatattaac-3′	391
8-9	5′-ttccatctgaag-cttttattgggaga-3′	392
9-10	5′-cctcaacaatgt-cttccccaacgtag-3′	393

Based on the sequences presented in table 4b, exon-bridging oligonucleotides for hIL1RI are designed with a length from 15 to 22 bases. These oligonucleotides have at least 4 nucleotides and at most 11 nucleotides at either 3' or 5' sequences adjacent to the exon-exon boundary between two exons. These oligonucleotides are presented in Table 3. GC content, Tm. For a few probes the similarity with other genes are indicated.

As shown in example 4, exon-bridging oligonucleotides which have more than 11 nucleotides at one or both ends of the exon-exon boundary [20 mer en 24 mer], and/or which have a length of more than 22 nucleotides [24 mer], still can inhibit hIL1RI expression.

Example 4: IL1RI antisense inhibition in chondrocytes.

Phenotypically stable human articular cartilage cells were obtained from normal and OA cartilage of the same knee showing focal OA. The cells were cultured in alginate beads over 1 week, as described in example 1 to re-establish the intracellular cytokine and growth factors levels, to re-express the respective plasma membrane receptors and to reach equilibrium in accumulated cell-associated matrix (CAM) compounds. Following liberation of the cells from the alginate beads, the levels of CAM aggrecan were assayed using flow cytometry as described in example 2.

When compared with cells obtained from normal cartilage tissues, the accumulation of aggrecan in the CAM was significantly reduced in the chondrocytes obtained from pathological tissue. This repression of CAM aggrecan was due to an increased activity of the catabolic IL1/IL1RI pathway in these cells, as illustrated an increased expression of IL1 and IL1RI on the plasma membrane of the OA chondrocytes, when compared with the cells obtained from normal tissues (see example 1 in this document). The chondrocytes were exposed to an IL1RI mRNA targeting exon bridging antisense oligonucleotide. A 15mer (probe 6; SEQ ID NO:6), 20mer (probe 7; SEQ ID NO 7) and 24 mer (probe 8, SEQ ID NO: 8) oligonucleotide was used as antisense oligonucleotide against IL1RI, all three bridging coding exons 2 and 3. All oligonucleotides had phosphorothioate modifications.

The efficacy of these oligonucleotide to silence IL1RI expression was tested on OA chondrocytes, as these cells were shown to express higher levels of this IL1 receptor (Table 4).

Table 4: Effect of IL1RI exon-bridging antisense oligonucleotides on IL1RI expression.

	15 mer [SEQ ID NO:6]			
	0 nM	1 nM	10 nM	100 nM
MFI for IL1RI	0.42 ± 0.04	0.48 ± 0.10	0.45 ± 0.15	0.14 ± 0.09
p-value		> 0.10	> 0.10	< 0.10
	20 mer [SEQ ID NO:7]			
	0 nM	1 nM	10 nM	100 nM
MFI for IL1RI	0.42 ± 0.04	0.28 ± 0.05	0.16 ± 0.10	0.12 ± 0.04
p-value		< 0.10	< 0.10	< 0.10

The chondrocytes in this experiment were obtained from a 64-year-old female. Results are expressed as Mean Fluorescence Intensity (MFI) for IL1RI of the cell population following staining of the cells with the FITC-labelled anti human IL1RI mAb, and after exposure to different concentrations (nM) of the antisense oligonucleotide. Experiments were performed in triplicate. Mean and 1SD are given. Statistical analysis was performed using the Student's T-Test.

The 20 mer antisense oligonucleotide [SEQ ID NO : 7] efficiently suppressed the expression of plasma membrane IL1RI. The 15mer [SEQ ID NO: 6] IL1RI antisense oligonucleotide showed less effects. Although the 20 in Table 4 is more effective at higher concentrations, 1 nM concentrations of the probe inhibited plasma membrane IL1RI expression with 33.4%.

Example 5 : Reversal of repression of the accumulation of aggrecan in the CAM of the OA chondrocytes.

Aggrecan accumulation was assayed after administration of the 20mer IL1RI antisense oligonucleotide [SEQ ID NO: 7] on OA chondrocytes obtained from 3 different patients (Table 5). For comparison, normal chondrocytes were obtained from one of these donors.

The administration of this antisense oligonucleotide had no effect on the aggrecan accumulation of chondrocytes from normal cartilage. However, repression of the accumulation of aggrecan in the CAM of the osteoarthritic chondrocytes was completely reversed when the 20 mer antisense oligonucleotide was added to these cells in culture. The effect was significant and dose-related when the oligonucleotide was added to a final concentration in the nutrient medium ranging from 1.0 to 100.0 nM.

Table 5: Effect of IL1RI antisense oligonucleotides on CAM aggrecan synthesis of chondrocytes

Donor F64 OA	0 nM	1 nM	10 nM	100 nM
MFI for aggrecan	100 ± 0.0	128.9 ± 6.8	128.2 ± 3.1	142.1 ± 2.1
p-value		< 0.10	< 0.10	< 0.10
Donor M69 OA	0 nM	1 nM	10 nM	100 nM
MFI for aggrecan	100 ± 0.0	121.1 ±19.8	127.8 ±18.5	133.0 ±17.5
p-value		< 0.10	< 0.10	< 0.10
Donor M60 OA	0 nM	1 nM	10 nM	100 nM
MFI for aggrecan	100 ± 0.0	117.6 ± 4.5	120.8 ± 4.5	139.4 ± 5.0
p-value		< 0.10	< 0.10	< 0.10
Donor M60 - NO	0 nM	1 nM	10 nM	100 nM
MFI for aggrecan	100 ± 0.0	97.4 ± 3.2	98.3 ± 5.2	102.1 ± 3.1
p-value		> 0.10	> 0.10	> 0.10

Experiments performed on the chondrocytes obtained from 3 different donors. Sex (F/M) and donor age are given. Results expressed as Mean Fluorescence Intensity (MFI) of the cell population after exposure to different concentrations (1-100 nM) of the antisense oligonucleotide. Control values were normalised to 100%. Experiments were performed in triplicate. Mean and 1SD are given. Statistics : Student's t-test.

Example 6 : Reversal of repression of the accumulation of aggrecan in the CAM of OA chondrocytes.

Further experiments were performed to evaluate the effects of 9 different exon-bridging probes corresponding to the 9 exon-bridging sequences in the IL1R1 mRNA. A comparison was made of the effects of exon-bridging and non-exon-bridging probes, i.e. antisense probes to sequences flanking the 5'-end of the corresponding exon bridge ('exon-flanking' probes, Figure 5). Moreover, a comparison was made with the effect of a non-exon bridging probe of the prior art, i.e. ISIS 8807 (described in US Patent No. 5,856,099).

The sequence of the oligomers used in this experiment are provided in Table 6.

1 nM concentrations were used. All tests were done in triplicate. The accumulation of aggrecan in the CAM of the chondrocytes in culture (MFI values obtained by flow cytometry) was used as a variable. The ISIS 8807 probe was used in comparison at a 1 nM concentration.

Table 6 : probes used

EBR	'flanking' probes	SEQ-ID/probe		exon-bridging probes	SEQ-ID/probe
01-02	5-ttatttttttcttcacgttcc-3	29	vs.	5'-ttgcatttat-cagcctccag-3'	20
02-03	5-attttaattctgaggcagta-3	30	vs.	5'-agatgaattt-cttaccacgc-3'	21
03-04	5-tatattgtcaagaagtagag-3	31	vs.	5'-gtttgcaatc-cttataccac-3'	22
04-05	5-caatcacaggccttgtgggt-3	32	vs.	5'-ttgttttcct-ctagagtaat-3'	23
05-06	5-tgacattacagatcaattgt-3	33	vs.	5'-atctgggatc-ccaagtctac-3'	24
06-07	5-ctccttcttttgtttgcagg-3	34	vs.	5'-attttccaca-ctgtaatagt-3'	25
07-08	5-taccaatcatgtgcttctgg-3	35	vs.	5'-aaattagtga-ctggatatat-3'	26
08-09	5-tatatgcgtcataggtcttt-3	36	vs.	5'-ccatctgaag-cttttattgg-3'	27
09-10	5-ttacgttttcattaatgacc-3	37	vs.	5'-tcaacaatgt-cttccccaac-3'	28

ISIS 8807: 5-tgtgtccaatcggtggc (directed against 3'UTR) (SEQ ID NO:38)

In order to obtain the percentage increases in MFI (cell-bound aggrecan) due to the effects of the different antisense sequences, the values for the control cultures were normalized to 100. A number of exon-bridging sequences were found to inhibit IL1R1 similarly or more significantly than their non-exon bridging counterparts. The aggrecan synthesis of the cells was significantly increased for both the oligomers corresponding to the exon 02-03 and 05-06. More particularly, from these experiments it could be deduced that the exon-bridging sequences eb02-03 (SEQ ID 21) and eb05-06 (SEQ ID 24) are particularly suited for IL1R1 inhibition.

The ISIS 8807 antisense sequence was not effective at the concentrations used.

Table 3: Potential exon-bridging oligomers

SEQ ID NOs are listed after the sequences in square brackets

A. EXON 01→02 exon-bridging oligomers

5'-CCTTGCATTTAT-CAGCCTCCAGAGAA-3' [76]

15mer exon-spanning probes
position 133-147; 134-148; 135-149; 136-150; 137-151; 138-152; 139-153; 140-154

probe	GC content	Tm
5'-TTAT-CAGCCTCCAGA-3' [39]	07/15=46.7%	44
5'-TTTAT-CAGCCTCCAG-3' [40]	07/15=46.7%	44
5'-ATTTAT-CAGCCTCCA-3' [41]	06/15=40.0%	42
5'-CATTTAT-CAGCCTCC-3' [42]	07/15=46.7%	44
5'-GCATTTAT-CAGCCTC-3' [43]	07/15=46.7%	44
5'-TGCATTTAT-CAGCCT-3' [44]	06/15=40.0%	42
5'-TTGCATTTAT-CAGCC-3' [45]	06/15=40.0%	42
5'-CTTGCATTTAT-CAGC-3' [46]	06/15=40.0%	42

16 mer exon-spanning probes
position 133-148; 134-149; 135-150; 136-151; 137-152; 138-153; 139-154

probe	GC content	Tm
5'-TTTAT-CAGCCTCCAGA-3' [47]	07/16=43.8%	46
5'-ATTTAT-CAGCCTCCAG-3' [48]	07/16=43.8%	46
5'-CATTTAT-CAGCCTCCA-3' [49]	07/16=43.8%	46
5'-GCATTTAT-CAGCCTCC-3' [50]	08/16=50.0%	48
5'-TGCATTTAT-CAGCCTC-3' [51]	07/16=43.8%	46
5'-TTGCATTTAT-CAGCCT-3' [52]	06/16=37.5%	44
5'-CTTGCATTTAT-CAGCC-3' [53]	07/16=43.8%	46

17 mer exon-spanning probes
position 133-149; 134-150; 135-151; 136-152; 137-153; 138-154

probe	GC content	Tm
5'-ATTTAT-CAGCCTCCAGA-3' [54]	07/17=42.2%	48
5'-CATTTAT-CAGCCTCCAG-3' [55]	08/17=47.1%	50
5'-GCATTTAT-CAGCCTCCA-3' [56]	08/17=47.1%	50
5'-TGCATTTAT-CAGCCTCC-3' [57]	08/17=47.1%	50
5'-TTGCATTTAT-CAGCCTC-3' [58]	07/17=42.2%	48
5'-CTTGCATTTAT-CAGCCT-3' [59]	07/17=42.2%	48

Table 3: Potential exon-bridging oligomers (continued)

18 mer exon-spanning probes
position 133-150; 134-151; 135-152; 136-153; 137-154

probe	GC content	Tm
5'-CATTTAT-CAGCCTCCAGA-3' [60]	08/18=44.4%	52
5'-GCATTTAT-CAGCCTCCAG-3' [61]	09/18=50.0%	54
5'-TGCATTTAT-CAGCCTCCA-3' [62]	08/18=44.4%	52
5'-TTGCATTTAT-CAGCCTCC-3' [63]	08/18=44.4%	52
5'-CTTGCATTTAT-CAGCCTC-3' [64]	08/18=44.4%	52

19 mer exon-spanning probes
position 133-151; 134-152; 135-153; 136-154

probe	GC content	Tm
5'-GCATTTAT-CAGCCTCCAGA-3' [65]	09/19=47.4%	56
5'-TGCATTTAT-CAGCCTCCAG-3' [66]	09/19=47.4%	56
5'-TTGCATTTAT-CAGCCTCCA-3' [67]	08/19=42.1%	54
5'-CTTGCATTTAT-CAGCCTCC-3' [68]	09/19=47.4%	56

20 mer exon-spanning probes
position 133-152; 134-153; 135-154

probe	GC content	Tm
5'-TGCATTTAT-CAGCCTCCAGA-3' [69]	09/20=45.0%	58
5'-TTGCATTTAT-CAGCCTCCAG-3' [70]	09/20=45.0%	58
5'-CTTGCATTTAT-CAGCCTCCA-3' [71]	09/20=45.0%	58

21 mer exon-spanning probes
position 133-153 ; 134-154

probe	GC content	Tm
5'-TTGCATTTAT-CAGCCTCCAGA-3' [72]	09/21=42.9%	60
5'-CTTGCATTTAT-CAGCCTCCAG-3' [73]	10/21=47.6%	62

22 mer exon-spanning probe
position 133-154

probe	GC content	Tm
5'-CTTGCATTTAT-CAGCCTCCAGA-3' [74]	10/22=45.5%	64°C

25 mer exon-spanning probe
position 131-155

probe	GC content	Tm
5'-CCTTGCATTTAT-CAGCCTCCAGAGA-3' [75]	12/25=48.0%	74°C

Table 3: Potential exon-bridging oligomers (continued)

B. EXON 02→03 exon-bridging oligomers

5′-AGTAAGATGAATTT-CTTACCACGCAA-3′ [77]

15 mer exon-spanning probes
position 368-382; 369-383; 370-384; 371-385; 372-386; 373-387; 374-388; 375-389

probe	GC content	Tm
5′-ATTT-CTTACCACGCA-3′ [78]	06/15=40.0%	42
5′-AATTT-CTTACCACGC-3′ [79]	06/15=40.0%	42
5′-GAATTT-CTTACCACG-3′ [80]	06/15=40.0%	42
5′-TGAATTT-CTTACCAC-3′ [81]	05/15=33.3%	40
5′-ATGAATTT-CTTACCA-3′ [82]	04/15=26.7%	38
5′-GATGAATTT-CTTACC-3′ [83]	05/15=33.3%	40
5′-AGATGAATTT-CTTAC-3′ [84]	04/15=26.7%	38
5′-AAGATGAATTT-CTTA-3′ [85]	03/15=20.0%	36

16 mer exon-spanning probes
position 368-383; 369-384; 370-385; 371-386; 372-387; 373-388; 374-389

probe	GC content	Tm
5′-AATTT-CTTACCACGCA-3′ [86]	06/16=37.5%	44
5′-GAATTT-CTTACCACGC-3′ [87]	07/16=43.8%	46
5′-TGAATTT-CTTACCACG-3′ [88]	06/16=37.5%	44
5′-ATGAATTT-CTTACCAC-3′ [89]	05/16=31.5%	42
5′-GATGAATTT-CTTACCA-3′ [90]	05/16=31.5%	42
5′-AGATGAATTT-CTTACC-3′ [91]	05/16=31.5%	42
5′-AAGATGAATTT-CTTAC-3′ [92]	04/16=25.0%	40

17 mer exon-spanning probes
position 368-384; 369-385; 370-386; 371-387; 372-388; 373-389

probe	GC content	Tm
5′-GAATTT-CTTACCACGCA-3′ [93]	07/17=41.2%	48
5′-TGAATTT-CTTACCACGC-3′ [94]	07/17=41.2%	48
5′-ATGAATTT-CTTACCACG-3′ [95]	06/17=35.3%	46
5′-GATGAATTT-CTTACCAC-3′ [96	06/17=35.3%	46
5′-AGATGAATTT-CTTACCA-3′ [97]	05/17=29.4%	44
5′-AAGATGAATTT-CTTACC-3′ [98]	05/17=29.4%	44

Table 3: Potential exon-bridging oligomers (continued)

18 mer exon-spanning probes
position 368-385; 369-386; 370-387; 371-388; 372-389

probe	GC content	Tm
5'-TGAATTT-CTTACCACGCA-3' [99]	07/18=38.9%	50
5'-ATGAATTT-CTTACCACGC-3' [100]	07/18=38.9%	50
5'-GATGAATTT-CTTACCACG-3' [101]	07/18=38.9%	50
5'-AGATGAATTT-CTTACCAC-3' [102]	06/18=35.3%	48
5'-AAGATGAATTT-CTTACCA-3' [103]	05/18=27.8%	46

19 mer exon-spanning probes
position 368-386; 369-387; 370-388; 371-389

probe	GC content	Tm
5'-ATGAATTT-CTTACCACGCA-3' [104]	07/19=36.8%	52
5'-GATGAATTT-CTTACCACGC-3' [105]	08/19=42.1%	54
5'-AGATGAATTT-CTTACCACG-3' [106]	07/19=36.8%	52
5'-AAGATGAATTT-CTTACCAC-3' [107]	06/19=31.6%	50

20 mer exon-spanning probes
position 368-387; 369-388; 370-389

probe	GC content	Tm
5'-GATGAATTT-CTTACCACGCA-3' [108]	08/20=40.0%	56
5'-AGATGAATTT-CTTACCACGC-3' [109]	08/20=40.0%	56
5'-AAGATGAATTT-CTTACCACG-3' [110]	07/20=35.0%	54

21 mer exon-spanning probes
position 368-388; 369-389

probe	GC content	Tm
5'-AGATGAATTT-CTTACCACGCA-3' [111]	08/21=38.1%	58
5'-AAGATGAATTT-CTTACCACGC-3' [112]	08/21=38.1%	58

22 mer exon-spanning probes
position 368-389

probe	GC content	Tm
5'-AAGATGAATTT-CTTACCACGCA-3' [113]	08/22=36.4%	60

25 mer exon-spanning probe
position 368-392

probe	GC content	Tm
5'-AGTAAGATGAATTT-CTTACCACGCAA-3' [114]	09/25=36.0%	68

Table 3: Potential exon-bridging oligomers (continued)

C. EXON 03→04 exon-bridging oligomers

5'-GAGGTTTGCAATC-CTTATACCACTGT-3' [115]

15 mer exon-spanning probes
position 558-572; 559-573; 560-574; 561-575; 562-576; 563-577; 564-578; 565-579

probe	GC content	Tm
5'-AATC-CTTATACCACT-3' [116]	05/15=33.3%	40
5'-CAATC-CTTATACCAC-3' [117]	06/15=40.0%	42
5'-GCAATC-CTTATACCA-3' [118]	06/15=40.0%	42
5'-TGCAATC-CTTATACC-3' [119]	06/15=40.0%	42
5'-TTGCAATC-CTTATAC-3' [120]	05/15=33.3%	40
5'-TTTGCAATC-CTTATA-3' [121]	04/15=26.7%	38
5'-GTTTGCAATC-CTTAT-3' [122]	05/15=33.3%	40
5'-GGTTTGCAATC-CTTA-3' [123]	06/15=40.0%	42

16 mer exon-spanning probes
position 558-573; 559-574; 560-575; 561-576; 562-577; 563-578; 564-579

probe	GC content	Tm
5'-CAATC-CTTATACCACT-3' [124]	05/16=33.3%	42
5'-GCAATC-CTTATACCAC-3' [125]	06/16=40.0%	44
5'-TGCAATC-CTTATACCA-3' [126]	06/16=40.0%	44
5'-TTGCAATC-CTTATACC-3' [127]	06/16=40.0%	44
5'-TTTGCAATC-CTTATAC-3' [128]	05/16=33.3%	42
5'-GTTTGCAATC-CTTATA-3' [129]	04/16=26.7%	40
5'-GGTTTGCAATC-CTTAT-3' [130]	05/16=33.3%	42

17 mer exon-spanning probes
position 558-574; 559-575; 560-576; 561-577; 562-578; 563-579

probe	GC content	Tm
5'-GCAATC-CTTATACCACT-3' [131]	07/17=42.2%	48
5'-TGCAATC-CTTATACCAC-3' [132]	07/17=42.2%	48
5'-TGCAATC-CTTATACCA-3' [133]	06/17=35.3%	46
5'-TTTGCAATC-CTTATACC-3' [134]	06/17=35.3%	46
5'-GTTTGCAATC-CTTATAC-3' [135]	06/17=35.3%	46
5'-GGTTTGCAATC-CTTATA-3' [136]	06/17=35.3%	46

Table 3: Potential exon-bridging oligomers (continued)

18 mer exon-spanning probes
position 558-575; 559-576; 560-577; 561-578; 562-579

probe	GC content	Tm
5'-TGCAATC-CTTATACCACT-3' [137]	07/18=42.2%	50
5'-TTGCAATC-CTTATACCAC-3' [138]	07/18=42.2%	50
5'-TTTGCAATC-CTTATACCA-3' [139]	06/18=35.3%	48
5'-GTTTGCAATC-CTTATACC-3' [140]	06/18=35.3%	48
5'-GGTTTGCAATC-CTTATAC-3' [141]	06/18=35.3%	48

19 mer exon-spanning probes
position 558-576; 559-577; 560-578; 561-579

probe	GC content	Tm
5'-TTGCAATC-CTTATACCACT-3' [142]	07/19=36.8%	52
5'-TTTGCAATC-CTTATACCAC-3' [143]	07/19=36.8%	52
5'-GTTTGCAATC-CTTATACCA-3' [144]	07/19=36.8%	52
5'-GGTTTGCAATC-CTTATACC-3' [145]	08/19=42.1%	54

20 mer exon-spanning probes
position 558-577; 559-578; 560-579

probe	GC content	Tm
5'-TTTGCAATC-CTTATACCACT-3' [146]	07/20=35.0%	54
5'-GTTTGCAATC-CTTATACCAC-3' [147]	08/20=40.0%	56
5'-GGTTTGCAATC-CTTATACCA-3' [148]	08/20=40.0%	56

21 mer exon-spanning probes
position 558-578; 559-579

probe	GC content	Tm
5'-GTTTGCAATC-CTTATACCACT-3' [149]	08/21=38.1%	58°C 6
5'-GGTTTGCAATC-CTTATACCAC-3' [150]	09/21=42.9%	60°C 9

22 mer exon-spanning probes
position 558-579

probe	GC content	Tm
5'-GGTTTGCAATC-CTTATACCACT-3' [151]	09/22=40.9%	62°C 10

Table 3: Potential exon-bridging oligomers (continued)

22 mer exon-spanning probes
position 559-581

probe	GC content	Tm
5'-GAGGTTTGCAATC-CTTATACCACTG-3' [152]	11/25=44.0%	72°C

D. EXON 04→05 exon-bridging oligomers

5'-GTTTGTTTTCCTC-TAGAGTAATAAAT-3' [153]

15 mer exon-spanning probes
position 727-741; 728-742; 729-743; 730-744; 731-745; 732-746; 733-747; 734-748

probe	GC content	Tm
5'-TCCT-CTAGAGTAATA-3' [154]	05/15=33.3%	40
5'-TTCCT-CTAGAGTAAT-3' [155]	05/15=33.3%	40
5'-TTTCCT-CTAGAGTAA-3' [156]	05/15=33.3%	40
5'-TTTTCCT-CTAGAGTA-3' [157]	05/15=33.3%	40
5'-GTTTTCCT-CTAGAGT-3' [158]	06/15=40.0%	42
5'-GTTTTCCT-CTAGAGT-3' [159]	06/15=40.0%	42
5'-TTGTTTTCCT-CTAGA-3' [160]	05/15=33.3%	40
5'-TTTGTTTTCCT-CTAG-3' [161]	05/15=33.3%	40

16 mer exon-spanning probes
position 727-742; 728-743; 729-744; 730-745; 731-746; 732-747; 733-748

probe	GC content	Tm
5'-TTCCT-CTAGAGTAATA-3' [162]	05/16=31.3%	42
5'-TTTCCT-CTAGAGTAAT-3' [163]	05/16=31.3%	42
5'-TTTTCCT-CTAGAGTAA-3' [164]	05/16=31.3%	42
5'-GTTTTCCT-CTAGAGTA-3' [165]	06/16=37.5%	44
5'-TGTTTTCCT-CTAGAGT-3' [166]	06/16=37.5%	44
5'-TTGTTTTCCT-CTAGAG-3' [167]	06/16=37.5%	44
5'-TTTGTTTTCCT-CTAGA-3' [168]	05/16=31.3%	42

17 mer exon-spanning probes
position 727-743; 728-744; 729-745; 730-746; 731-747; 732-748

probe	GC content	Tm
5'-TTTCCT-CTAGAGTAATA-3' [169]	05/17=29.4%	44
5'-TTTTCCT-CTAGAGTAAT-3' [170]	05/17=29.4%	44
5'-GTTTTCCT-CTAGAGTAA-3' [171]	06/17=35.3%	46
5'-TGTTTTCCT-CTAGAGTA-3' [172]	06/17=35.3%	46

Table 3:Potential exon-bridging oligomers (continued)

5'-TTGTTTTCCT-CTAGAGT-3'[173]	06/17=35.3%	46
5'-TTTGTTTTCCT-CTAGAG-3'[174]	06/17=35.3%	46

18 mer exon-spanning probes
position 727-744; 728-745; 729-746; 730-747; 731-748

probe	GC content	Tm
5'-TTTTCCT-CTAGAGTAATA-3'[175]	05/18=27.8%	46
5'-GTTTTCCT-CTAGAGTAAT-3'[176]	06/18=33.3%	48
5'-TGTTTTCCT-CTAGAGTAA-3'[177]	06/18=33.3%	48
5'-TTGTTTTCCT-CTAGAGTA-3'[178]	06/18=33.3%	48
5'-TTTGTTTTCCT-CTAGAGT-3'[179]	06/18=33.3%	48

19 mer exon-spanning probes
position 727-745; 728-746; 729-747; 730-748

probe	GC content	Tm
5'-GTTTTCCT-CTAGAGTAATA-3'[180]	06/19=31.6%	50
5'-TGTTTTCCT-CTAGAGTAAT-3'[181]	06/19=31.6%	50
5'-TTGTTTTCCT-CTAGAGTAA-3'[182]	06/19=31.6%	50
5'-TTTGTTTTCCT-CTAGAGTA-3'[183]	06/19=31.6%	50

20 mer exon-spanning probes
position 727-746; 728-747; 729-748

probe	GC content	Tm
5'-TGTTTTCCT-CTAGAGTAATA-3'[184]	06/20=30.0%	52
5'-TTGTTTTCCT-CTAGAGTAAT-3'[185]	06/20=30.0%	52
5'-TTTGTTTTCCT-CTAGAGTAA-3'[186]	06/20=30.0%	52

21 mer exon-spanning probes
position 727-747; 728-748

probe	GC content	Tm
5'-TTGTTTTCCT-CTAGAGTAATA-3'[187]	06/21=28.6%	54
5'-TTTGTTTTCCT-CTAGAGTAAT-3'[188]	06/21=28.6%	54

22 mer exon-spanning probes
position 727-749

probe	GC content	Tm	
5'-TTTGTTTTCCT-CTAGAGTAATA-3'[189]	06/22=27.3%	56°	45

NB5'-12mer

Table 3: Potential exon-bridging oligomers (continued)

25 mer exon-spanning probes
position 725-749

probe	GC content	Tm
5'-GTTTGTTTTCCT-CTAGAGTAATAAA-3' [190]	07/25=28.0%	64%

E. EXON 05→06 exon-bridging oligomers

5'-GTATCTGGGATC-CCAAGTCTACTTCC-3' [191]

15 mer exon-spanning probes
position 793-807; 794-808; 795-809; 796-810; 797-811; 798-812; 799-813; 800-814

probe	GC content	Tm
5'-GATC-CCAAGTCTACT-3' [192]	07/15=46.7%	44
5'-GGATC-CCAAGTCTAC-3' [193]	08/15=53.3%	46
5'-GGGATC-CCAAGTCTA-3' [194]	08/15=53.3%	46
5'-TGGGATC-CCAAGTCT-3' [195]	08/15=53.3%	46
5'-CTGGGATC-CCAAGTC-3' [196]	09/15=60.0%	48
5'-TCTGGGATC-CCAAGT-3' [197]	08/15=53.3%	46
5'-ATCTGGGATC-CCAAG-3' [198]	08/15=53.3%	46
5'-TATCTGGGATC-CCAA-3' [199]	07/15=46.7%	44

16 mer exon-spanning probes
position 793-808; 794-809; 795-810; 796-811; 797-812; 798-813; 799-814

probe	GC content	Tm
5'-GGATC-CCAAGTCTACT-3' [200]	08/16=50.0%	48
5'-GGGATC-CCAAGTCTAC-3' [201]	09/16=56.3%	50
5'-TGGGATC-CCAAGTCTA-3' [202]	08/16=50.0%	48
5'-CTGGGATC-CCAAGTCT-3' [203]	09/16=56.3%	50
5'-TCTGGGATC-CCAAGTC-3' [204]	09/16=56.3%	50
5'-ATCTGGGATC-CCAAGT-3' [205]	08/16=50.0%	48
5'-TATCTGGGATC-CCAAG-3' [206]	08/16=50.0%	48

17 mer exon-spanning probes
position 793-809; 794-810; 795-811; 796-812; 797-813; 798-814

probe	GC content	Tm
5'-GGGATC-CCAAGTCTACT-3' [207]	09/17=52.9%	52
5'-TGGGATC-CCAAGTCTAC-3' [208]	09/17=52.9%	52
5'-CTGGGATC-CCAAGTCTA-3' [209]	09/17=52.9%	52
5'-TCTGGGATC-CCAAGTCT-3' [210]	09/17=52.9%	52

Table 3: Potential exon-bridging oligomers (continued)

5'-ATCTGGGATC-CCAAGTC-3' [211]	09/17=52.9%	52
5'-TATCTGGGATC-CCAAGT-3' [212]	08/17=47.1%	50

18 mer exon-spanning probes
position 793-810; 794-811; 795-812; 796-813; 797-814

probe	GC content	Tm
5'-TGGGATC-CCAAGTCTACT-3' [213]	09/18=50.0%	54
5'-CTGGGATC-CCAAGTCTAC-3' [214]	10/18=55.5%	56
5'-TCTGGGATC-CCAAGTCTA-3' [215]	09/18=50.0%	54
5'-ATCTGGGATC-CCAAGTCT-3' [216]	09/18=50.0%	54
5'-TATCTGGGATC-CCAAGTC-3' [217]	09/18=50.0%	54

19 mer exon-spanning probes
position 793-811; 794-812; 795-813; 796-814

probe	GC content	Tm
5'-CTGGGATC-CCAAGTCTACT-3' [218]	10/19=52.6%	58
5'-TCTGGGATC-CCAAGTCTAC-3' [219]	10/19=52.6%	58
5'-ATCTGGGATC-CCAAGTCTA-3' [220]	09/19=47.4%	56
5'-TATCTGGGATC-CCAAGTCT-3' [221]	09/19=47.4%	56

20 mer exon-spanning probes
position 793-812; 794-813; 795-814

probe	GC content	Tm
5'-TCTGGGATC-CCAAGTCTACT-3' [222]	10/20=50.0%	60
5'-ATCTGGGATC-CCAAGTCTAC-3' [223]	10/20=50.0%	60
5'-TATCTGGGATC-CCAAGTCTA-3' [224]	09/20=45.0%	58

21 mer exon-spanning probes
position 793-813; 794-814

probe	GC content	Tm
5'-ATCTGGGATC-CCAAGTCTACT-3' [225]	10/21=47.6%	62
5'-TATCTGGGATC-CCAAGTCTAC-3' [226]	10/21=47.6%	62

22 mer exon-spanning probes
position 793-814

probe	GC content	Tm
5'-TATCTGGGATC-CCAAGTCTACT-3' [227]	10/22=45.5%	64

Table 3: Potential exon-bridging oligomers (continued)

25 mer exon-spanning probes
position 791-815

probe	GC content	Tm
5'-GTATCTGGGATC-CCAAGTCTACTTC-3'[228]	12/25=48.0%	74

F. EXON 06→07 exon-bridging oligomers

5'-CAGGATTTTCCACA-CTGTAATAGTCT-3'[229]

15 mer exon-spanning probes
position 911-925; 912-926; 913-927; 914-928; 915-929; 916-930; 917-931; 918-932

probe	GC content	Tm
5'-CACA-CTGTAATAGTC-3'[230]	06/15=40.0%	42
5'-CCACA-CTGTAATAGT-3'[231]	06/15=40.0%	42
5'-TCCACA-CTGTAATAG-3'[232]	06/15=40.0%	42
5'-TTCCACA-CTGTAATA-3'[233]	05/15=33.0%	40
5'-TTTCCACA-CTGTAAT-3'[234]	05/15=33.3%	40
5'-TTTTCCACA-CTGTAA-3'[235]	05/15=33.3%	40
5'-ATTTTCCACA-CTGTA-3'[236]	05/15=33.3%	40
5'-GATTTTCCACA-CTGT-3'[237]	06/15=40.0%	42

16 mer exon-spanning probes
position 911-926; 912-927; 913-928; 914-929; 915-930; 916-931; 917-932

probe	GC content	Tm
5'-CCACA-CTGTAATAGTC-3'[238]	07/16=43.8%	46
5'-TCCACA-CTGTAATAGT-3'[239]	06/16=37.5%	44
5'-TTCCACA-CTGTAATAG-3'[240]	06/16=37.5%	44
5'-TTTCCACA-CTGTAATA-3'[241]	05/16=31.3%	42
5'-TTTTCCACA-CTGTAAT-3'[242]	05/16=31.3%	42
5'-ATTTTCCACA-CTGTAA-3'[243]	05/16=31.3%	42
5'-GATTTTCCACA-CTGTA-3'[244]	06/16=37.5%	44

17 mer exon-spanning probes
position 911-927; 912-928; 913-929; 914-930; 915-931; 916-932

probe	GC content	Tm
5'-TCCACA-CTGTAATAGTC-3'[245]	07/17=41.2%	48
5'-TTCCACA-CTGTAATAGT-3'[246]	06/17=35.3%	46
5'-TTTCCACA-CTGTAATAG-3'[247]	06/17=35.3%	46
5'-TTTTCCACA-CTGTAATA-3'[248]	05/17=29.4%	44

Table 3: Potential exon-bridging oligomers (continued)

5'-ATTTTCCACA-CTGTAAT-3' [249]	05/17=29.4%	44
5'-GATTTTCCACA-CTGTAA-3' [250]	06/17=35.3%	46

18 mer exon-spanning probes
position 911-928; 912-929; 913-930; 914-931; 915-932

probe	GC content	Tm
5'-TTCCACA-CTGTAATAGTC-3' [251]	07/18=38.9%	50
5'-TTTCCACA-CTGTAATAGT-3' [252]	06/18=33.3%	48
5'-TTTTCCACA-CTGTAATAG-3' [253]	06/18=33.3%	48
5'-ATTTTCCACA-CTGTAATA-3' [254]	05/18=27.8%	46
5'-GATTTTCCACA-CTGTAAT-3' [255]	06/18=33.3%	48

19 mer exon-spanning probes
position 911-929; 912-930; 913-931; 914-932

probe	GC content	Tm
5'-TTTCCACA-CTGTAATAGTC-3' [256]	07/19=36.8%	52
5'-TTTTCCACA-CTGTAATAGT-3' [257]	06/19=31.6%	50
5'-ATTTTCCACA-CTGTAATAG-3' [258]	06/19=31.6%	50
5'-GATTTTCCACA-CTGTAATA-3' [259]	06/19=31.6%	50

20 mer exon-spanning probes
position 911-930; 912-931; 913-932

probe	GC content	Tm
5'-TTTTCCACA-CTGTAATAGTC-3' [260]	07/20=35.0%	54
5'-ATTTTCCACA-CTGTAATAGT-3' [261]	06/20=30.0%	52
5'-GATTTTCCACA-CTGTAATAG-3' [262]	07/20=35.0%	54

21 mer exon-spanning probes
position 911-931; 912-932

probe	GC content	Tm
5'-ATTTTCCACA-CTGTAATAGTC-3' [263]	07/21=33.3%	56
5'-GATTTTCCACA-CTGTAATAGT-3' [264]	07/21=33.3%	56

22 mer exon-spanning probes
position 911-932

probe	GC content	Tm
5'-GATTTTCCACA-CTGTAATAGTC-3' [265]	08/22=40.9%	60°C 46

25 mer exon-spanning probes
position 911-935

Table 3: Potential exon-bridging oligomers (continued)

probe	GC content	Tm
5'-CAGGATTTTCCACA-CTGTAATAGTC-3' [266]	10/25=40.0%	70°C

G. EXON 07→08 exon-bridging oligomers

5'-GGAAATTAGTGA-CTGGATATATTAAC-3' [267]

15 mer exon-spanning probes
position 1063-1077; 1064-1078; 1065-1079; 1066-1080; 1067-1081; 1068-1082; 1069-1083; 1070-1084

probe	GC content	Tm
5'-GTGA-CTGGATATATT-3' [268]	05/15=33.3%	40
5'-AGTGA-CTGGATATAT-3' [269]	05/15=33.3%	40
5'-TAGTGA-CTGGATATA-3' [270]	05/15=33.3%	40
5'-TTAGTGA-CTGGATAT-3' [271]	05/15=33.3%	40
5'-ATTAGTGA-CTGGATA-3' [272]	05/15=33.3%	40
5'-AATTAGTGA-CTGGAT-3' [273]	05/15=33.3%	40
5'-AAATTAGTGA-CTGGA-3' [274]	05/15=33.3%	40
5'-GAAATTAGTGA-CTGG-3' [275]	06/15=40.0%	42

16 mer exon-spanning probes
position 1063-1078; 1064-1079; 1065-1080; 1066-1081; 1067-1082; 1068-1083; 1069-1084

probe	GC content	Tm
5'-AGTGA-CTGGATATATT-3' [276]	05/16=31.3%	42
5'-TAGTGA-CTGGATATAT-3' [277]	05/16=31.3%	42
5'-TTAGTGA-CTGGATATA-3' [278]	05/16=31.3%	42
5'-ATTAGTGA-CTGGATAT-3' [279]	05/16=31.3%	42
5'-AATTAGTGA-CTGGATA-3' [280]	05/16=31.3%	42
5'-AAATTAGTGA-CTGGAT-3' [281]	05/16=31.3%	42
5'-GAAATTAGTGA-CTGGA-3' [282]	06/16=37.5%	44

17 mer exon-spanning probes
position 1063-1079; 1064-1080; 1065-1081; 1066-1082; 1067-1083; 1068-1084

probe	GC content	Tm
5'-TAGTGA-CTGGATATATT-3' [283]	05/17=29.4%	44
5'-TTAGTGA-CTGGATATAT-3' [284]	05/17=29.4%	44
5'-ATTAGTGA-CTGGATATA-3' [285]	05/17=29.4%	44
5'-AATTAGTGA-CTGGATAT-3' [286]	05/17=29.4%	44
5'-AAATTAGTGA-CTGGATA-3' [287]	05/17=29.4%	44
5'-GAAATTAGTGA-CTGGAT-3' [288]	06/17=35.3%	46

Table 3: Potential exon-bridging oligomers (continued)

18 mer exon-spanning probes
position 1063-1080; 1064-1081; 1065-1082; 1066-1083; 1067-1084

probe	GC content	Tm
5'-TTAGTGA-CTGGATATATT-3' [289]	05/18=27.8%	46
5'-ATTAGTGA-CTGGATATAT-3' [290]	05/18=27.8%	46
5'-AATTAGTGA-CTGGATATA-3' [291]	05/18=27.8%	46
5'-AAATTAGTGA-CTGGATAT-3' [292]	05/18=27.8%	46
5'-GAAATTAGTGA-CTGGATA-3' [293]	06/18=33.3%	48

19 mer exon-spanning probes
position 1063-1081; 1064-1082; 1065-1083; 1066-1084

probe	GC content	Tm
5'-ATTAGTGA-CTGGATATATT-3' [294]	05/19=26.3%	48
5'-AATTAGTGA-CTGGATATAT-3' [295]	05/19=26.3%	48
5'-AAATTAGTGA-CTGGATATA-3' [296]	05/19=26.3%	48
5'-GAAATTAGTGA-CTGGATAT-3' [297]	06/19=31.6%	50

20 mer exon-spanning probes
position 1063-1082; 1064-1083; 1065-1084

probe	GC content	Tm
5'-AATTAGTGA-CTGGATATATT-3' [298]	05/20=25.0%	50
5'-AAATTAGTGA-CTGGATATAT-3' [299]	05/20=25.0%	50
5'-GAAATTAGTGA-CTGGATATA-3' [300]	06/20=30.0%	52

21 mer exon-spanning probes
position 1063-1083; 1064-1084

probe	GC content	Tm
5'-AAATTAGTGA-CTGGATATATT-3' [301]	05/21=23.8%	52
5'-GAAATTAGTGA-CTGGATATAT-3' [302]	06/21=28.6%	54

22 mer exon-spanning probes
position 1063-1084

probe	GC content	Tm
5'-GAAATTAGTGA-CTGGATATATT-3' [303]	06/22=27.3%	56°C 29

25 mer exon-spanning probes
position 1061-1085

probe	GC content	Tm
5'-GGAAATTAGTGA-CTGGATATATTAA-3' [304]	07/25=28.0%	64°C

Table 3: Potential exon-bridging oligomers (continued)

H. EXON 08→09 exon-bridging oligomers

5'-TTCCATCTGAAG-CTTTTATTGGGAGA-3' [305]

15 mer exon-spanning probes
position 1207-1221; 1208-1222; 1209-1223; 1210-1224; 1211-1225; 1212-1226; 1213-1227; 1214-1228

probe	GC content	Tm
5'-GAAG-CTTTTATTGGG-3' [306]	06/15=40.0%	42
5'-TGAAG-CTTTTATTGG-3' [307]	05/15=33.3%	40
5'-CTGAAG-CTTTTATTG-3' [308]	05/15=33.3%	40
5'-TCTGAAG-CTTTTATT-3' [309]	04/15=26.7%	38
5'-ATCTGAAG-CTTTTAT-3' [310]	04/15=26.7%	38
5'-CATCTGAAG-CTTTTA-3' [311]	05/15=33.3%	40
5'-CCATCTGAAG-CTTTT-3' [312]	06/15=40.0%	42
5'-TCCATCTGAAG-CTTT-3' [313]	06/15=40.0%	42

16 mer exon-spanning probes
position 1207-1222; 1208-1223; 1209-1224; 1210-1225; 1211-1226; 1212-1227; 1213-1228

probe	GC content	Tm
5'-TGAAG-CTTTTATTGGG-3' [314]	06/16=37.5%	44
5'-CTGAAG-CTTTTATTGG-3' [315]	06/16=37.5%	44
5'-TCTGAAG-CTTTTATTG-3' [316]	05/16=31.3%	42
5'-ATCTGAAG-CTTTTATT-3' [317]	04/16=25.0%	40
5'-CATCTGAAG-CTTTTAT-3' [318]	05/16=31.3%	42
5'-CCATCTGAAG-CTTTTA-3' [319]	06/16=37.5%	44
5'-TCCATCTGAAG-CTTTT-3' [320]	06/16=37.5%	44

17 mer exon-spanning probes
position 1207-1223; 1208-1224; 1209-1225; 1210-1226; 1211-1227; 1212-1228

probe	GC content	Tm
5'-CTGAAG-CTTTTATTGGG-3' [321]	07/17=41.2%	48
5'-TCTGAAG-CTTTTATTGG-3' [322]	06/17=35.3%	46
5'-ATCTGAAG-CTTTTATTG-3' [323]	05/17=29.4%	44
5'-CATCTGAAG-CTTTTATT-3' [324]	05/17=29.4%	44
5'-CCATCTGAAG-CTTTTAT-3' [325]	06/17=35.3%	46
5'-TCCATCTGAAG-CTTTTA-3' [326]	06/17=35.3%	46

18 mer exon-spanning probes
position 1207-1224; 1208-1225; 1209-1226; 1210-1227; 1211-1228

Table 3: Potential exon-bridging oligomers (continued)

probe	GC content	Tm
5'-TCTGAAG-CTTTTATTGGG-3' [327]	06/18=33.3%	48
5'-ATCTGAAG-CTTTTATTGG-3' [328]	06/18=33.3%	48
5'-CATCTGAAG-CTTTTATTG-3' [329]	06/18=33.3%	48
5'-CCATCTGAAG-CTTTTATT-3' [330]	06/18=33.3%	48
5'-TCCATCTGAAG-CTTTTAT-3' [331]	06/18=33.3%	48

19 mer exon-spanning probes
position 1207-1225; 1208-1226; 1209-1227; 1210-1228

probe	GC content	Tm
5'-ATCTGAAG-CTTTTATTGGG-3' [332]	07/19=36.8%	52
5'-CATCTGAAG-CTTTTATTGGA-3' [333]	07/19=36.8%	52
5'-CCATCTGAAG-CTTTTATTG-3' [334]	07/19=36.8%	52
5'-TCCATCTGAAG-CTTTTATT-3' [335]	06/19=31.6%	50

20 mer exon-spanning probes
position 1207-1226; 1208-1227; 1209-1228

probe	GC content	Tm
5'-CATCTGAAG-CTTTTATTGGG-3' [336]	08/20=40.0%	56
5'-CCATCTGAAG-CTTTTATTGG-3' [337]	08/20=40.0%	56
5'-TCCATCTGAAG-CTTTTATTG-3' [338]	07/20=35.0%	54

21 mer exon-spanning probes
position 1207-1227; 1208-1228

probe	GC content	Tm
5'-CCATCTGAAG-CTTTTATTGGG-3' [339]	09/21=42.9%	60
5'-TCCATCTGAAG-CTTTTATTGG-3' [340]	08/21=38.1%	58

22 mer exon-spanning probes
position 1207-1228

probe	GC content	Tm
5'-TCCATCTGAAG-CTTTTATTGGG-3' [341]	09/22=40.9%	62°C 39

25 mer exon-spanning probes
position 1205-1229

probe	GC content	Tm
5'-TTCCATCTGAAG-CTTTTATTGGGAG-3' [342]	10/25=40.0%	70°C

Table 3: Potential exon-bridging oligomers (continued)

I. EXON 09→10 exon-bridging oligomers

5'-CCTCAACAATGT-CTTCCCCAACGTAG-3' [343]

15 mer exon-spanning probes
position 1375-1389; 1376-1390; 1377-1391; 1378-1392; 1379-1393; 1380-1394; 1381-1395; 1382-1396

probe	GC content	Tm
5'-ATGT-CTTCCCCAACG-3' [344]	08/15=53.3%	46
5'-AATGT-CTTCCCCAAC-3' [345]	07/15=46.7%	44
5'-CAATGT-CTTCCCCAA-3' [346]	07/15=46.7%	44
5'-ACAATGT-CTTCCCCA-3' [347]	07/15=46.7%	44
5'-AACAATGT-CTTCCCC-3' [348]	07/15=46.7%	44
5'-CAACAATGT-CTTCCC-3' [349]	07/15=46.7%	44
5'-TCAACAATGT-CTTCC-3' [350]	06/15=40.0%	42
5'-CTCAACAATGT-CTTC-3' [351]	06/15=40.0%	42

16 mer exon-spanning probes
position 1375-1390; 1376-1391; 1377-1392; 1378-1393; 1379-1394; 1380-1395; 1381-1396

probe	GC content	Tm
5'-AATGT-CTTCCCCAACG-3' [352]	08/16=50.0%	48
5'-CAATGT-CTTCCCCAAC-3' [353]	08/16=50.0%	48
5'-ACAATGT-CTTCCCCAA-3' [354]	07/16=43.8%	46
5'-AACAATGT-CTTCCCCA-3' [355]	07/16=43.8%	46
5'-CAACAATGT-CTTCCCC-3' [356]	08/16=50.0%	48
5'-TCAACAATGT-CTTCCC-3' [357]	07/16=43.8%	46
5'-CTCAACAATGT-CTTCC-3' [358]	07/16=43.8%	46

17 mer exon-spanning probes
position 1375-1391; 1376-1392; 1377-1393; 1378-1394; 1379-395; 1380-1396

probe	GC content	Tm
5'-CAATGT-CTTCCCCAACG-3' [359]	09/17=52.9%	52
5'-ACAATGT-CTTCCCCAAC-3' [360]	08/17=47.1%	50
5'-AACAATGT-CTTCCCCAA-3' [361]	07/17=41.2%	48
5'-CAACAATGT-CTTCCCCA-3' [362]	08/17=47.1%	50
5'-TCAACAATGT-CTTCCCC-3' [363]	08/17=47.1%	50
5'-CTCAACAATGT-CTTCCC-3' [364]	08/17=47.1%	50

18 mer exon-spanning probes
position 1375-1392; 1376-1393; 1377-1394; 1378-1395; 1379-1396

Table 3: Potential exon-bridging oligomers (continued)

probe	GC content	Tm
5'-ACAATGT-CTTCCCCAACG-3' [365]	09/18=50.0%	54
5'-AACAATGT-CTTCCCCAAC-3' [366]	08/18=44.4%	52
5'-CAACAATGT-CTTCCCCAA-3' [367]	08/18=44.4%	52
5'-TCAACAATGT-CTTCCCCA-3' [368]	08/18=44.4%	52
5'-CTCAACAATGT-CTTCCCC-3' [369]	09/18=50.0%	54

19 mer exon-spanning probes
position 1375-1393; 1376-1394; 1377-1395; 1378-1396

probe	GC content	Tm
5'-AACAATGT-CTTCCCCAACG-3' [370]	09/19=47.4%	56
5'-CAACAATGT-CTTCCCCAAC-3' [371]	09/19=47.4%	56
5'-TCAACAATGT-CTTCCCCAA-3' [372]	08/19=42.1%	54
5'-CTCAACAATGT-CTTCCCCA-3' [373]	09/19=47.4%	56

20 mer exon-spanning probes
position 1375-1394; 1376-1395; 1377-1396

probe	GC content	Tm
5'-CAACAATGT-CTTCCCCAACG-3' [374]	10/20=50.0%	60
5'-TCAACAATGT-CTTCCCCAAC-3' [375]	09/20=45.5%	58
5'-CTCAACAATGT-CTTCCCCAA-3' [376]	09/20=45.5%	58

21 mer exon-spanning probes
position 1375-1395; 1376-1396

probe	GC content	Tm
5'-TCAACAATGT-CTTCCCCAACG-3' [377]	10/21=47.6%	62
5'-CTCAACAATGT-CTTCCCCAAC-3' [378]	10/21=47.6%	62

22 mer exon-spanning probes
position 1375-1396

probe	GC content	Tm
5'-CTCAACAATGT-CTTCCCCAACG-3' [379]	11/22=50.0%	66°C

25 mer exon-spanning probes
position 1373-1397

probe	GC content	Tm
5'-CCTCAACAATGT-CTTCCCCAACGTA-3' [380]	12/25=48.0%	74°C

Note: "T" and "A" 5' of the exon-exon boundary of exons 02→03 and 06→07 do not match the genomic sequence, they match M27492.